HISTOLOGICAL AND HISTOCHEMICAL STUDIES OF CERTAIN ORGANS IN DIFFERENT AGE GROUPS OF *Channa punctatus* WITH SPECIAL REFERENCE TO LIPOFUSCIN PIGMENTS

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Dedicated To My Dear Parents

DECLARATION

I hereby declare that with the exceptions of the guidence and suggestions received from my supervisor, Dr. R.C.Gupta, Reader, Post Graduate Department of Zoology, Bipin Bihari College, Jhansi, this thesis is my own unaided work carried out in the Post Graduate Department of Zoology, Bipin Bihari College, Jhansi, U.P.

I further declare that this is an original piece of my research work and has not been submitted any where else.

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CERTIFICATE

This is to certify that the thesis entitled " *Histological and histochemical studies of certain organs in different age groups of Channa punctatus with special reference to lipofuscin*." is an original piece of research work carried out by **Mr. Vimlesh Kumar Agarwal,** in the Deptt. of Zoology, Bipin Bihari P.G. College, Jhansi. He has worked under my guidence and supervision for the period (more than 200 days) required as under para 8 of the Ph. D. ordinance. In my opinion this thesis fulfils the requirements of the ordinance relating to the Ph.D. Degree of Bundelkhand University Jhansi.

Date : 10th Oct. 95

(R.C. Gupta)

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CONTENTS

CHAPTER - 1

INDRODUCTION

Aging is a definite time course and direction for the individual changes in various parts of an organism. It is an universal phenomenon in all the organism, the total of which results in the failure of individual to withstand the stress of his environment. Obvious manifestation of aging includes wrinkling of skin, slowness of movement & inability of the eyes to accomadate for near vision. Another manifestation of aging is the wide spread accumulation of pigment granules within the cytoplasm of cells of many organs particularly in neurones, skeletal, myocardial, carpus luteum, spleen, liver and nerve cells. A consistantly noted change in cell's composition during aging is the increase of substance variously known as aging pigment, age pigment, lipofuscin, ceroid or wear and tear pigment. The structure and composition of pigment may vary from species to species and tissues to tissues.

The involvement of this pigment in aging process has been the subject of much study and controversy. The pigment has been recognised as a distinct intracellular structure for over a century, (Hannover 1842, Koneff 1886). Hannover (1842) observed to these pigments in dissected nerve cells whereas Koneff (1886) reported that the amount of these pigment granules in nerve cells are related to the age of individual. Hodge (1894) reported pigment accumulation

in the cytoplasm of neurones of senile individual as well as in honeybee and human nerve cells. Stubel (1911) observed these pigments particularly in brain. Hueck (1912) reported the occurrence of fluorescent inclusion in cells which generally referred as lipofuscin or age pigment. Pinkerton (1928) suggested the importance of unsaturated lipids in the biogenesis of lipofuscin and Muhlmann (1910) stress the significance of pigmentation in nerve cells with age.

Koneff (1886) was the first to associate the presence of pigment granules with cellular aging. This hypothesis is now universally accepted so much, so that of the cytological modifications corelated to neuronal aging. The progressive accumulation of lipofuscin is considered to be the most indicative. Fluorescent pigment granules were found in the electric lobes of Torpedo, (Lerma & Ventra, 1956),and these were later associated to lipofuscin pigments; (Totaro, 1977).

It is known for over a century that the cytoplasm of aging nerve cells of human and other animals species contain golden brown pigment inclusions called lipofuscin (Whiteford & Getty, 1966).

The complexity of the pigment is evidenced by voluminous litrature which has accumulated during this period. According to Connor (1928), two groups of pigments accumulate in the body with age namely lipochrome and haemochrome. The former is exogenious in origin and is found normally in such

organs as the adrenal cortex, carpus leteum, liver, spleen and skin, while later is the wear and tear pigment and has been found in many organs including heart.

The term lipofuscin was proposed by Borst(1922), however other terms that have been assigned to the insoluble yellow intracellular material are, lipochrome (Findley; 1920, Bourne; 1934, Fekete; 1946), wear and tear pigment (Hemperl; 1934), luleolipin (Rossman; 1942), ceroid (Reagan; 1950), lemofuscin (Jayne; 1950), yellow pigment (Hyden & Lindstorm; 1950), lipogene pigment (Gomori; 1952), lipopigment (Gedigk & Bontke; 1956), age pigment (Strehler et al ; 1959, Brody; 1960) and lipid pigment (Samorajski & Ordy; 1967), but the term lipofuscin has been accepted by several investigators (Bensley; 1947, Strehler et al; 1959, Pearse; 1960, Frank & Christensen; 1968).

Numerous investigators have observed accumulation of a yellow brown pigment in certain body cells of various aging animals. The most regular cytological change corelated with aging is the accumulation of a type of pigment of lipid origin commonly known as lipofuscin (Bensley; 1947).

Publications by Bjorkerud (1964), Strehler (1964) and Goldfischer, et al (1966) reported the informations available concerning the biochemical composition, histochemical properties and the ultrastructural morphology of these pigments.

Some of the pigment granules found in the various tissues have been thought to owe their origin to the process of aging. This assumption seems to be justified since pigments are not normally found in young animals but are seen in the tissues of old animals.

Occurrence of lipofuscin pigment was known as early as the end of the last century. Since then, the origin and possible function of lipofuscin granules have been the subject of much interest. Histochemical studies in situ, fluorescent microscopy and recently electron microscopy have yielded no definite conclusion regarding its origin. However, the origin of lipofuscin has been attributed to many of the cytoplasmic organelles including mitochondria, golgi apparatus, endoplasmic reticulum and lysosomes. (Toth; 1968)

Mitochondria generally found in association with the pigment and electron micrographs of dorsal root ganglia from aged mice have indicated the possibility of pigment formation from them (Duncan et al ; 1960). In a study of neurosecretion in birds (Ghosh et al ; 1962) have obtained some interesting electron micrographs of mitochondria and lipofuscin which further support mitochondrial origin of age pigment. Examinations by light and electron microscopy of human myocardium have revealed that mitochondria can be transformed into granules of lipofuscin (Koobs et al ; 1978). Certain other workers have reported that lipofuscin is derived from degenerating mitochondria (Hess;

1955, Glees and Gopinath; 1973, Spoerri and Glees; 1973, Gopinath andGlees; 1974).

Gatenby & Mausa (1950) and Gatenby (1953) established a morphological relationship between the golgi apparatus and lipofuscin. Mitochondrial origin of the pigment was strongly disputed by Bondareff (1957,1959), who suggested a possible relationship of pigment to the golgi apparatus. Another possibility may be the involvement of endoplasmic reticulum in formation of lipofuscin. (Chance & Williams; 1954, Kumamoto and Bourne; 1963)

Support for a mitochondrial source of age pigment or lipofuscin, however clashes to some extent and several groups of workers have suggested an identity of lipofuscin with lysosome. De Duve et al (1955) provided concerning evidence for structural and cytochemical identification of lipofuscin with lysosome in nerve cells. According to Samorajski et al (1964) lipofuscin represents the remains of lysosomes. Findings of Gedigk and Bontke (1956), Essener and Novikoff (1960), Hassan and Glees (1972) and Brunk and Ericsson (1972) are particularly relevant to this hypothesis.

Current theories on the nature and origin of these pigments are however based largely on in vitro experiments (Chio & Tappel; 1969, Chio et al ; 1969, Fletcher, Dillard & Tappel; 1973, Esterbauer, et al ; 1991) and on the

properties and composition of isolated fluorescent granules, organic solvent extracts, and detergents solubilized preparations. Isolated cells and tissues slides have also been studied using fluorescent microscopy (Totaro, Glees and Pisanti, 1985; Harman, 1990; Porta, 1991). Results obtained using these methods may not describe the fluorescent properties of only those materials that increase with age. Clearly, limited purification can result in isolation of fluorescent material and cellular components unrelated to lipofuscin and may lead to erroneous conclusions as to the composition and derivation of these pigments (Csallany & Ayaz, 1976). Before the role of lipofuscin in aging can be clarified, the chemical nature and origin of age pigments must be determined. Complete characterization requires purification and direct chemical analysis of fluorophores that increase as a function of age.

The most obvious and consistent evidence of the aging process at the cellular level is the accumulation of lipofuscin pigments. Although there have been many studies on the composition and origin of these aging or wear and tear pigments much is yet unknown of lipofuscin real link to the process of cellular aging.

Generally thought to be a result of polyunsaturated lipid peroxidation in a number of biological membranes such as mitochondria, microsomes and lysosomes (Chio et al , 1969; Tappel, 1975; Sanadi, 1977), lipofuscin exhibits a

wide variety of physical characteristics that make the pigment simple to identify if confusing to comprehendfully. One property that has proven especially useful in lipofuscin identification is its yellow-gold antofluorescence when exposed to ultra violet and near ultra violet light, (Hyden & Lindstrom, 1950; Strehler et al ; 1959, Csallany & Ayaz, 1976:)

Lipofuscin is general term assigned to fluorescent material that accumulates in cells as they aged, while reports on the fluorescent properties vary, this material typically emits light between 450 nm and 600 nm. Fluorescence described to lipofuscin has been observed in post mitotic cells from a variety of organisms within intracellular granules composed, in part, of protein and lipid. (Totaro, Glees & Pisanti, 1985; Zs Nagy, 1988; Tsuchida, Miura & Aibara, 1987; Harman, 1990; Porta, 1991). Because of its age related increase and seemingly universal occurrence lipofuscin is considered a hall mark of aging.

Lipofuscin has been located in cytoplasm in the groups of various sizes and also collected from the perikaryon or at the poles of cells. Histochemical studies (Bourne, 1973) have clarified, but not defined the composition of lipofuscin while the genesis and functional significance of these masses of pigments are still a matter of discussion. It is widely held that these granules are either of a mitochondrial (Miquel, 1971; Gopinath & Glees, 1974; Glees, Hasan &

Spoerri, 1974) or a lysosomal (Samorajski & Ordy, 1972; Hasan & Glees, 1972,1973) derivatives. Regarding the functional significance it is suggested that lipofuscin is cellular debris waiting to be eliminated. (Gless and Hasan, 1976)

Scattered histochemical work have been carried out on the different enzymes associated with lipofuscin in senile animals. (Lillie, 1950; Gedick & Bontke, 1956; Shanklin & Nassar, 1957; Gomori, 1958)

Lipofuscin has been proposed as a basic "LAW OF SENESCENCE" for cellular aging (Strehler & Barrow, 1970). However the early appearance and experimental modifications of lipofuscin by drugs, harmones, antioxidants and immunoregulators have suggested to others that the pigments may represents a harmless or innocuous byproduct of cellular metabolism. (Nandy, 1968; Kormenday & Bender, 1971)

Since genetic and environmental interactions on the rate of lipofuscin accumulation with age can be clarified primarily in carefully controlled lifespan studies, increasing attention has been directed towards the morphological features,

intracellular origin, chemical composition and possible functional significance of lipofuscin in neurons, glea, neuropil and other extraneuronal constituents of the brain. (Friede, 1966; Ordy & Schjeide, 1973)

Aging pigments may be derived from a number of lipids or lipoprotein sources and the process by which they are produced is at least an oxidation (pearse, 1960).

Bensley (1947), demonstrated the pigments in the mitochondria of guinea pig's liver cells and compared these with those produced by the autooxidation of phospholipid and unsaturated fats. Lipofuscin is mainly lipoproteic in nature it increases with age so much so that it is known as "SENESCENCE PIGMENT". (Bourne, 1973; Glees & Hasan, 1976). Lipofuscin is widely believed to be a high molecular weight material generated upon oxidative damage of cellular components. (Tsuchida, Miura & Aibara, 1987; Kikugawa & Beppu, 1987; Harman, 1990; Porta, 1991; Ivy et al , 1991)

Lipofuscin granules have been defined as intracellular entities characterized by pigmentation, autofluorescence when irradiated by near ultraviolet light, partial insolubility in lipid solvents, positive PAS, and schmorl's reactions acid fastness and stain ability with Sudan black B. (Bjorkerud, 1964)

Efforts to determine the chemical nature of lipofuscin by means of the light microscope and special histochemical techniques have revealed that lipofuscin is PAS positive (Sulkin, & Kuntz, 1952; Sulkin, 1953, 1955a), stains with lipid stains (Sulkin, 1955a; Issidorides & Shanklin, 1961;) is occasionally acidfast (Pearse, 1960), gives a positive schmorl's reaction and reduces silver salts (Barka

& Anderson, 1962). With near ultra-violet light, lipofuscin granules exhibit a strong orange autofluorescence in unstained sections (Samorajski, Keefe & Ordy, 1964).

No single histochemical method however has identified this pigment, indicating its heterogeneous chemical compositions. Various histochemical test revealed that lipofuscin contains lipid, corbohydrate and protein (Handley et al , 1963; Bjorkerud, 1964a). Lipofuscin stains with carbol fuchsin, ferricferri cyanide, methyl green, PAS, Sudan black B and Nile blue A (Wolf & Pappenheimer, 1945; Lillie,1950,1956; Dixon & Herbertson, 1950; Alpert et al , 1960; Strehler, 1964; Sharma, 1967). Nandy (1971), demonstrated some interesting results which indicated the presence of two types of lipofuscin with different physical and chemical properties in neurons of mice of different ages. According to him these two types of lipofuscin pigments found in young and old mice vary in respect of their distribution, stainability, solubility, enzyme activity and fluorescence properties. Strehler (1964), reported that a typical section stained with Sudan black B was essentially similar in appearance to the sections stained with Nile blue sulphate. Interesting results on the binding of basic dyes have been obtained with Long-Ziehl-Neelsen's acid fast stain (Fewlgen et al , 1929;Pearse, 1960). In this procedure lipofuscin is essentially the only component in the tissue giving a positive response.

Vitamine E seems to play an important role in the central nervous

system with regard to metabolic stability and physiological functions (Nelson et al , 1981). Although there have been only a few studies on the effect of dietary Vitamine E on lipofuscin accumulation with age in the rats brain, the results have been inconsistent. (Katz, et al 1984; Sarter, 1987; Sato, 1988). Studies indicated that the rate of formation of lipofuscin pigment is increased in mice and rats by a Vitamine E deficient diet, (Sulkin & Samorajski, 1960; Reichel et al , 1968). Whereas supplementation of diets with Vitamine E decreased lipofuscin content in mice brain. (Freund, 1979)

Lipofuscin concentration can be reduced by treatment of centrophenoxine (Nandy & Bourne, 1966; Nandy et al , 1978). Hasan et al , (1974), demonstrated that centrophenoxine administered to guinea pigs for 30 to 56 days, caused removal of pigments from midbrain where as Spoerri and Glees(1975) suggested that after 70 days of drug administration, the number and size of pigment granules were greatly reduced. Alternatively, it may benefit the tissue by removing toxic substances from the cytoplasm. (Barber & Bernheim, 1967; Packer et al , 1967; Sinex, 1977; Miquel et al , 1978; Brizzee and Ordy, 1979) Strehler (1967), suggests that the accumulation of age pigment is an example of Stochastic Senescence. He further suggested that lipofuscin accumulation is the result of the evolutionary difficulty of selecting a sufficiently stable envelop which can content lysosomal enzymes, can be readily broken down during cellular reorganization and posseses stability against oxygen and

other reactants in the cell millieu.

Strehler et al (1959), have defined following criteria for biological aging.

a). UNIVERSALITY:- The change should occur universally in all old animals of a species and should be essentially absent in the very young.

b). TIME DEPENDENCE:- The change should progress gradually in an individual and in population.

c) INTRINSICALITY:- The change should be consequence of the action of time on the innate properties of the biological system rather then a result of a preventable disease, accident or pathology.

d) DELETERIOUSNESS:- The change should be unfavourable in its effect on the survival capacity of the individual organism in its normal environment, the change should be of such magnitude that it could contribute substantially to the functional ability of an organ and its host.

Among the intracellular changes occuring during the normal aging of human beings and other animals is the accumulation of dark brown lipid containing granules called lipofuscin (Hueck; 1912, Bommer;1929, Hamperl; 1934). This age pigment which posseses a bright yellow orange fluorescent

occurs primarily in non replaceable cells lines such as heart, brain and muscles and in the case of human myocardium it has been shown to accumulate linearly with age. (Strehler, et al 1959, Strehler & Mildvan, 1962)

One of the most consistent morphological observations made on tissue from aging animals is the accumulation of the intra cellular aging pigment (Timiras, 1972, Schofield and Davis 1978;). This material has been noted in a variety of cells over a period of a century or more on research (Strehler et al , 1959; Samorajski et al , 1964; Bourne, 1973;).

Research studies of lipofuscin have emphasized phyletic distribution , occurrence in human tissues, biophysical and biochemical properties, histochemical affinities and ultrastructure. Although various investigations have attempted to elicit the origin and function of lipofuscin. Results have implicated almost every cell organelle, that the accumulation of lipofuscin in non replaceable, fixed post mitotic cells is an age corelated process, is well established. (Jayne, 1950; Strehler et al . 1959; Brody, 1960). However it is indeed questionable, if lipofuscin is an age pigment resulting from a genetic program or the result of environmental influences i.e. biological noise.

Lipofuscin is known to accumulate in many tissues with increasing age of an organism as specially shown by histochemical staining, under light and electron microscope. (Samorajski & Ordy, 1967; Brody & Vijai Shankar, 1977;).

An extensive review by Szabo (1935) gives descriptions of age related morphological changes in many invertebrates from protozoans to insects, including the accumulation of age pigment as one indication of aging in these lower forms. Szabo (1935) also describe the accumulation of age pigment in ganglia and nerve cells of many different species of molluscs. Rudzianska (1961) reports this age pigment in protozoans cytoplasm, Epstein et al (1972) in nematodes, Totaro (1981) in Aplysia limacina, Sheehy et al (1991) in insects, crustaceans and other aquatic species and Fleming et al (1992) in Drosophilla.

Lipofuscin pigments accumulation has been observed to increase with age in human being (Brody, 1960; Samorajski et al , 1964), dogs (Whiteford & Getty, 1966; Few & Getty, 1967;), pigs (Whiteford & Getty, 1966; Few & Getty, 1967;Nanda & Getty, 1971), mice (Samorajski et al , 1968), rats (Reichel et al , 1968; Brizzee & Johnson, 1970) & monkeys (Brizzee et al , 1974). Mostly these studies has revealed the semi quantitative evidences for lipofuscin, increase with age, whereas Strehler et al (1959) & Goyal(1981) reported quantitatively that lipofuscin pigment granules accumulate linearly with time in human ventricular myocardium.

Lipofuscin pigment accumulation in the human heart has been observed to increase with age (Strehler et al , 1959; Mc Millan & Lev, 1962; Andrelej & Buozynski, 1972). Mc Millan & Lev (1962) reported an absence of

pigment in the myocardium of person below 10 yrs of age. With the advancement of age lipofuscin pigments has been observed to increase significantly in the ventricle of rat, (Reichel, 1968) and dog (Munnell and Getty 1968). There is no significant report available on the rate of lipofuscin pigment accumulation in human myocardium except that of Strehler et al , (1959).

By using fluorescent light microscope (Munnell & Getty, 1968) determined strong corelation betweem age and the amount of lipofuscin in myocardium of human and dogs. The granules accumulated faster in dogs than human, where as the life span of later is more than that of the former. Munnell & Getty (1968) suggested that this point has a meaningful connection between lipofuscin accumulation and life span. The linear relationship of accumulation of lipofuscin is supported by (Strehler et al , 1959) in man but children below 12 years of age did not possess the pigment. Quantitative observations made by (Goyal, 1981) also showed linear relationship between fluorescent pigment accumulation in myocardium with age in man.

The majority of investigators studying cardiac lipofuscin have been reported on its increasing incidence with age (Bohmig, 1935; Jayne, 1950; Strehler et al , 1959). Strehler et al , (1959) measured the amount of age pigment in microscopic section from hearts of humans, the study showed that the accumulation of the pigments increased linearly with age. Studies from laboratory

have been directed towards the relationship of lipofuscin to aging (Whiteford & Getty, 1966; Few & Getty, 1967).

Tissues of aged animals perticularly nervous tissues, adrenal gland, cardiac muscles and skeletal muscles are known to contain substantial amount of lipofuscin or age pigment. (Jayne,1950,1957,1963;Planel. & Guilhem, 1955; Meyers & Chariaper,1956; Strehler et al ,1959, Wilcox,1959; Mac Kinnon & Mac Kinnon 1960 Tcheng et al 1961; Whiteford & Getty, 1966).

Occurrence of interneuronal lipofuscin is established to be an age associated change in fishes, amphibians, birds, rats, pigs, dogs and man (Wilcox, 1959; Sulkin 1961; Whiteford & Getty, 1967; Singh & Mukherjee, 1972, Lopez et al 1993, Girven et al 1993, Kara 1994). Whiteford & Getty (1966) and Nanda & Getty (1971, 1973) reported the occurrence of lipofuscin and its increase amount with age in the nucleus, occulomotorii of pigs and dog. Hopker (1951)reported the presence of this pigment in the denate nucleus of the man. Large quantities of lipofuscin have been found in the central nervous system of Torpedo particularly in the electric lobes which are ganglia that regulate the function of eletric organs, (Totaro & Pisanti, 1981).

A number of studies on the aging pigment in nervous tissue, with regard to its occurrence, accumulation with age, its origin and significance, have been published,and these have been reviewed in detail by (Lansing,1952;

Birren,1959; Bourne, 1961; Barka and Anderson,1963; Whiteford, 1964; Few,1966,and Nanda,1970). The available records show that the occurrence of aging pigment in nervous tissue has been studied extensively in rat, mouse and guinea pig (Wilcox,1959). Several studies have been reported on human, (Andrew,1956; Bondareff,1959; Bourne,1961) dog, (Dolley,1911; Good Pasture,1918; Harms,1924; Sulkin & Kuntz,1952; Sulkin,1953, 1955a,1955b), where as only few studies available on the aging of nervous tissue of the pig, (Whiteford,1964; Few,1966)

By histochemical staining techniques and, light and electron microscopy an increase in pigment content with age has also been noted in the nervous system of mice (Samorajski, Keefe & Ordy, 1964; Samorajski, Ordy & Keefe, 1965) dogs (Sulkin, 1955; Whiteford & Getty, 1966), pigs (Whiteford & Getty, 1966) and human (Hamperl, 1934; Humphrey, 1944; Sulkin, 1953; Issidorides & Shanklin, 1961; Samorajski, et al . 1964), rats (Monji et al 1993). The most typical lipofuscin pigment is described by (Pearse,1960) as a brown deposite which is strongly basophilic and contains reducing moieties.

Gardner (1940) considered the presence of pigment in spinal ganglion cells to be related to age. Sulkin and Kuntz (1952) found the sympathetic ganglion cells to contain pigment granules in childrens and adults up to go years of age, but they are unable to demonstrate a progressive increase in pigment

concentration in human sympathetic ganglion cells with age. However,the presence of this material was frequently observed in dogs after 12 years of age,although pigments were not present in a siries of dogs aged 30 days to 9 years. On this basis these authors considered pigmentation to be definitely associated with aging in these animals.

Studies on the intracellular distribution of the pigment showed different patterns during aging. Hopker (1951) noted a variable distribution pattern relative to certain cell types and aging. Whiteford and Getty (1966) grouped the intracellular distribution of the pigment in various areas of the brain of dogs and pigs in to four categories: (a) Diffuse type granules, small and evenly scattered through out the cytoplasm; (b) Perinuclear pigment clusters, usually concentrated in a crescent shaped configuration; (c) Polar or axonal pigment aggregation, in which pigment was collected at or near the axon hillock; (d) Bipolar aggregation.

Cellular and regional differences have been reported in the accumulation of lipofuscin in the brain of man (Brody, 1960; Braak, 1971; Dayan, 1971). These findings have suggested that the deterioration of some sensory associative and motor function in senescence may be a consequence of selective alterations in discrete regions of the brain rather than uniform cellular aging throughout the nervous system.

The accumulation and changing pattern of distribution of lipoid pigment

bodies in neurones of the mammalian nervous system has been described by many investigators, (Dolley,1911; Harm,1924; Kuntz,1938; Nandy & Bourne,1966) Over a period of more than 80 yrs., among recent investigations Brody (1960) published data on the reletive amount and pattern of distribution of lipofuscin in neurones. Neurones exhibits lipofuscin pigment in both scattered and congregated distribution. Lipofuscin pigment diffused throughout the cytoplasm in early stages but may later be localized in perinuclear clusters or in polar aggregate of cells. (Brizzee, et al ,1969)

From the electron micrographs it has been determined that lipofuscin appears as clusters of high density and complex ultrastructure consisting of myelin like figures arranged in several configurations within a single body. (Samorajski, et al , 1964; Samorajski, Ordy & Keefe, 1965). The pigment bodies were reported to range in size from 2-3 microns were vocoulated, and were surrounded by a single limiting membrane (Samorajski et al , 1964; 1965). Observations with light microscope on the morphological features have shown the pigments as round or oblong granules increasing in size with age. (Brizzee et al , 1969)

Lipofuscin accumulation with aging mulnutrition and under various experimental conditions in the post mitotic cells of experimental animals and has been extensively studied and rewied. (Glees & Hasan,1976; Brizzee & Ordy,1981;

Patro, Sharma & Patro, 1988)

Whether lipofuscin is a harmful agent or an inert or harmless product is a debatable question.

Keeping in view the relationship of age and pigment accumulation the present study was planned to examine the earliest and gradual age dependent accumulation of lipofuscin pigment in different tissues of premature, mature & postmature, Channa punctatus:-

1. To establish the age at which lipofuscin pigment first appears in different tissues.

2. To examine possible differences in different organs and cytological distribution of lipofuscin pigment granules in different tissues in regard to the heart and brain.

3. To establish the progressive increase in the intra cellular pigment accumulation in various tissues.

4. To determine the morphology of pigment granules.

5. To evaluate the percentage of pigmented cells in heart and brain in different age groups.

CHAPTER - 2

Channna punctatus, a live fish is best suited for present investigation as it may be easily maintained under laboratory conditions for longer duration. The availability of Channa punctatus is round the year and can be procured on experimental requirements from natural habitate.

The fresh and reared fishes were dissected repeatedly in breeding season (July to Sept.) to observe their sexual maturity. The gonads, were taken out and actual state of maturity has been histologically studied in each of fish taken in the consideration for the present investigation.

Regarding the sexual maturity it is observed that Channa punctatus under primitive stage exhibits thin gonads, translucent pale in colour with inconspicuous vascularization. Histologically the ovary shows immature oocytes, while testes consists of small seminiferous tubules full with spermatogonia in formative phase.

In continuation when advanced (adult) fishes were examined, the gonads were found turgid, thicker, opaque, deep yellowish in colour and a large number of ova and sperms were visible, through ovarian and testicular cortex respectively. The gonads attain their maximum weight and volume, the fishes

become gravid due to ripe gametes and, abdomen become rounded and bulged.

Further when larger and older fishes were dissected, the gonads were found to flacid due to excessive dicharge of ova and sperms. The weight and volume was considerably reduced. Histologically, the ovaries show atretic dicharged follicles and testes show empty and collapsing seminiferous tubules.

On the basis of observations the experimental fishes were classified into three progressive age groups. i. e. premature, mature and post mature, respectively.

Under the matured condition fish exhibits morphological distinctive characteristics which were mainly noted during acute maturation of fish,are given as such for actual determination of fish Channa punctatus.

(1) Soft bulging and rounded abdomen.

(2) Bright colouration of the body.

(3) Fishes with prolonged fins & fin rays.

(4) Milt easily oozes out on pressing the abdomen.

FIXATION OF TISSUES

Studies are performed in considering the heart and brain of Channa punctatus. The heart is a triangnlar, pulsetile organ situated mid ventrally beneath

the pharynx within the pericardial cavity and, brain is white coloured, elongated and some what flattened sense organ lying well protected in side the cranium of skull.

The fishes from all the three age groups were dissected and, heart and brain were fixed in 10 % neutral formaline (90:10 water and formaline by volume). For histological and histochemical observations, representative tissues from different fishes of progressive age groups were fixed in small tubes for 4-6 days. (Brody, 1960; David et al, 1960; Nandy, 1968; Samorajski et al, 1968).

PREPARATION OF BOLCKS AND MICROTOMY

After fixing for 4-6 days all the tissues were dehydrated in graded alcoholic series in small pieces. After completion of 5 days the material was taken out from the mixture of 10 % neutral formalin and tissues were washed twice or thrice with running tap water and were then transformed in the alcoholic series. Materials were kept seperately at an interval of 10 minute in 30, 50, 70, 90 % and absolute alcohols, and thereafter they were passed through methyl benzoate, 3 changes of 10 minuteseach and cleared in benzene by 2 changes of 5 minutes. Finally, materials were kept in molten paraffin for 3 changes of 30 minutes at the 60 C and blocks were prepared. Series of sections were cut at 6 thickness and mounted on glass slides.

For qualitative studies, 25 slides of different tissues were prepared for

each age group. To determine the percentage of pigmented cells in the heart and brain of approximately equal size were selected randomly from histologic sections and were examined under light microscope. The mean of pigmented cells were calculated after examination of cells from ten selected stained slides. For quantitative estimation 20 cells per volume were examined from the heart and brain each.

HISTOCHEMICAL METHODS

Slides with mounted paraffin sections were deparaffinized in xylene and passed through the alcoholic series.

Histochemical studies were made in sections stained with following stains.

1. Nile Blue A (Lillie, 1956)

2. Schmorls ferric ferricyanide method (Pearse, 1960)

3. Carbol fuchsin, Long-Ziehl-Neelsen method (Pearse, 1960)

With all the above stains, granules were characterized by their affinities where as non granular deposition of cytoplasm remain unstained. According to Sharma and Manocha (1977), the pigment showed a characteristically differential staining behaviour. If, for instance one granule is moderately or intensively positive to a particular stain, other lying in its vicinity may be totally negative or

partially negative or partially positive. The variations may be due to differences in the composition of the lipofuscin.

Slides of tissues for different age groups were made by each stain.

1- NILE bLUE A (LILLIE'S ALTERNATIVE METHOD).

The stain was prepared from .05 % Nile blue A, in solution of 1 % sulphuric acid. The paraffin sections were deparaffinized and hydrated as usual, then stained for 20 minutes in .05 % Nile blue A stain. Stained slides were washed for 15 minutes in running water, mounted in glycerine jelly and examined under a light microscope. Lipofuscin pigment granules appeared as green-blue by this stain. Nuclei stained poorly or not at all. Due to temporary mount, the lipofuscin granules were photographed immidiately after preparation of slides.

2- FERRIC FERRICYANIDE ; (SCHMORL'S) METHOD

This method was used successfully by Sulkin (1955) Samorajski (1964) and Nandy (1968,1971). Ferric ferricyanide solution was prepared by 3 parts of 1% ferric chloride and 1 part of freshly prepared 1% potassium ferricyanide. Stain was always used within 30 minutes after preparation.

The paraffin sections of the heart and brain were deparaffinized, passed through alcoholic series and washed in water. Then the slides were

immeresed in ferric ferricyanide solution for 5 minutes, dipped in water 4 or 5 times and counter stained in 1% neutral red for 3 minutes. Stained slides were dehydrated rapidly in alcohol, cleared in xylene and mounted in DPX. Prepared slides of each tissue were examined under a light microscope. Lipofuscin pigment granules appeared dark blue whereas nucleus appeared red.

3- CARBOL FUCHSIN (LONG-ZIEHL-NEELSEN'S) METHOD

This method has also been used by several workers for acid fast lipofuscin (Sulkin 1955; Few & Getty 1967; Manocha & Sharma 1978; Nandy 1971). Carbol fuchsin solution was prepared as under :-

Basic fuchsin	---	10 gm.
Phenol	---	50 gm.
Alcohol	---	100 ml.
Distilled water	---	1000 ml.

Paraffin sections of the heart and brain were deparaffinized and hydrated as usual washed in water and stained in carbol fuchsin solution for 3 hrs. at 60 C. Sections were washed in running water and diffrentiated in 1% acid alcohol untill the red cells became just faint pink. Then sections were counter stained with .5% toludine blue for 50 minutes, again washed in running water

dehydrated in alcohol, cleared in xylene and mounted in DPX.

Lipofuscin pigment granules appeared bright red while nucleus was blue in colour.

Statistical analysis was followed after Snedecor (1957).

CHAPTER - 3

OBSERVATIONS AND RESULTS

In the present study, the intracellular distribution, morphology and accumulation of lipofuscin pigment in the heart and brain of Channa punctatus were investigated in three age groups i.e. premature, mature and postmature respectively.

The pigmented cells were observed in all the tissues (heart and brain) for quantitative and qualitative studies. Results obtained from the observations revealed the following four categories of cells in relation to the amount and distribution pattern of lipofuscin pigment granules.

CATEGORY -1 - Cells with no pigments.

CATEGORY - 2 - First or early appearance of pigments in very few cells.

CATEGORY - 3 - Cells containing homogeneous pigments throughout the cytoplasm.

CATEGORY - 4 - Cells with heterogeneous and clusters of pigment granules distributed throughout in the cytoplasm or at periphery of the cells or perinuclear in position.

Studies in relation to number of pigmented cells, structural morphology and distribution of lipofuscin, showed that intracytoplasmic location of pigment granules varies with age in different tissues and pigmented cells increased with age.

AGE GROUP- 1 :

The first age group includes premature Channa punctatus. Several paraffin sections of heart and brain were stained with Nile blue A , carbol fuchsin and ferric ferricyanide methods and were examined for early appearance of lipofuscin pigment granules.

HEART,

Observations from stained sections of heart of premature fishes have revealed that the lipofuscin pigments did not appear in cells. (Figs. 1,2). Nuclei were rounded and centrally located within the cells (Fig. 3), and non granular cytoplasmic substance had no affinities with any stain.

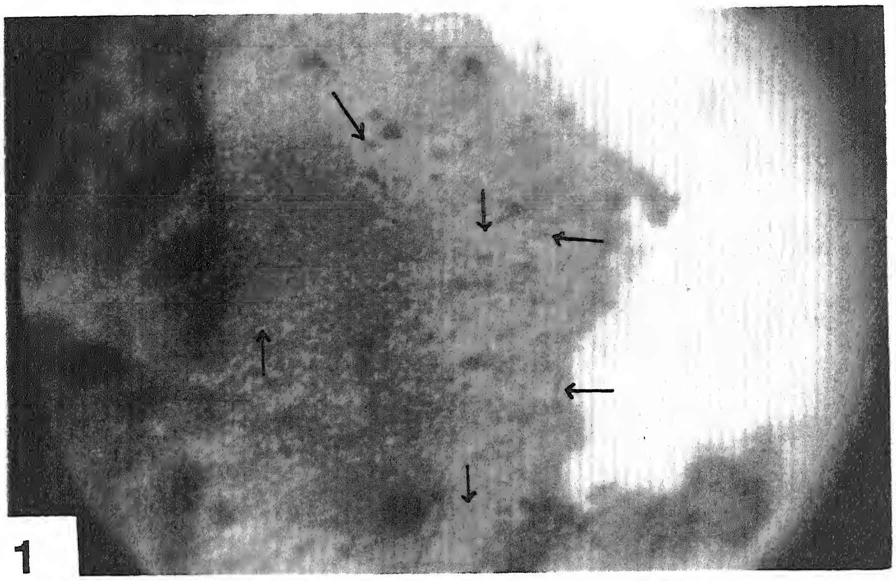
BRAIN,

Similarly the results of brain cells have revealed that the cytoplasmic substance stains lightly but lipofuscin granules did not appear in cytoplasm.(Figs. 4,5,6,7,8).

Results revealed that the cells of heart and brain of premature fishes show no pigment granules. While nuclei were found rounded and centrally placed within the cells.

Fig. 1 : Section of heart from premature fish.
Cells without lipofuscin pigments.
Nucleus (arrows) centrally placed
Carbol fuchsin stain x 450

Fig. 2 : Section of he: t from premature fish.
Cells without lipofuscin pigments.
Nucleus (arrows) centrally placed
Ferric ferricyanide stain x 450



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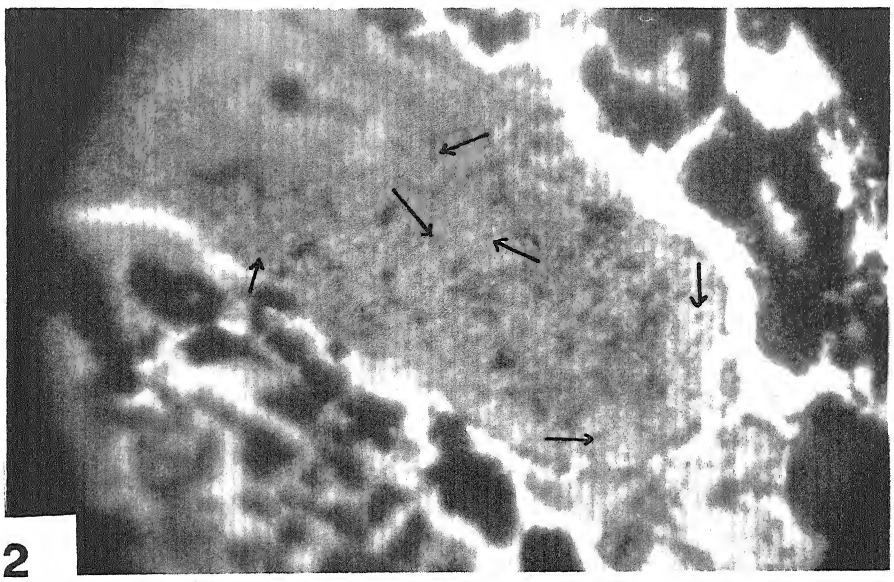

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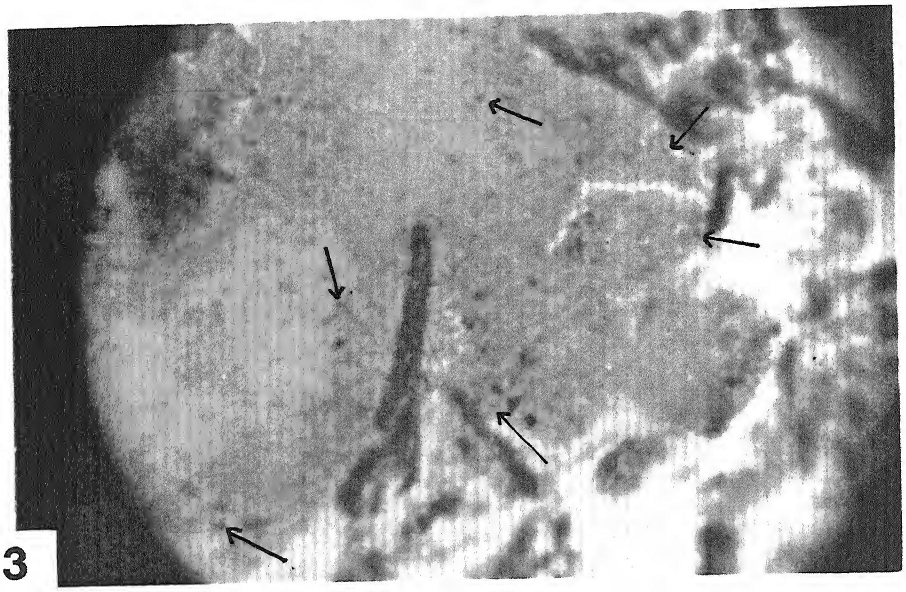
Fig. 3 : Section of heart from premature fish.
Cells showing no pigments, and rounded and centrally placed nuclei (arrows) appeared.
Nile blue A stain x 450

Fig. 4 : Section of brain from premature fish.
Cells with centrally placed nuclei (arrows) but lipofuscin pigments did not appear.
Ferric ferricyanide stain x 450



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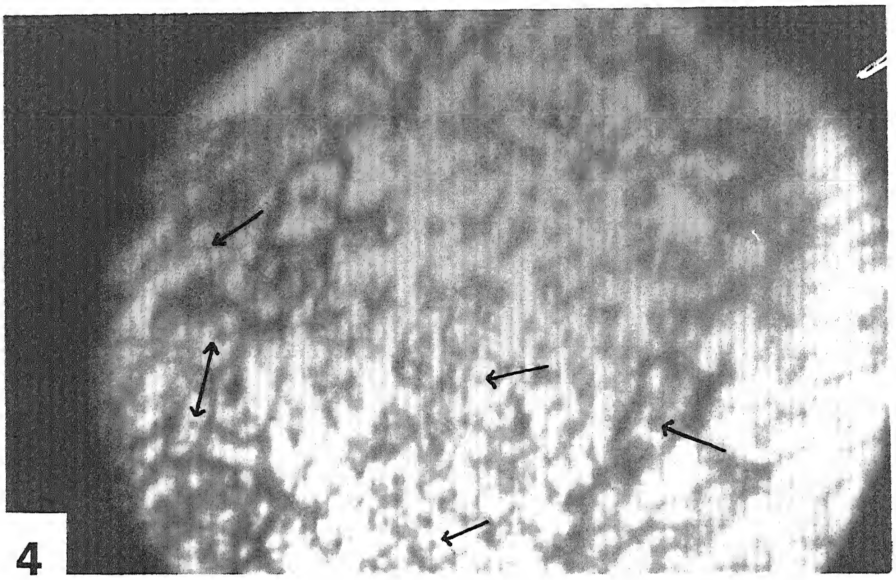

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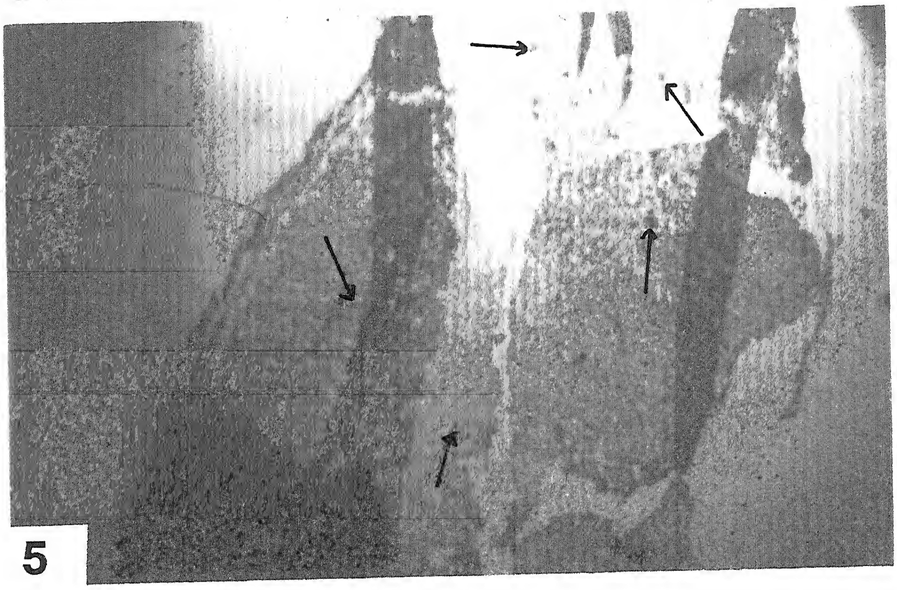
Fig. 5 : Section of brain from premature fish.
Cells showing no pigmentation.
Nucleus (arrows) has great affinity with stain.
Nile blue A stain x 450

Fig. 6 : Section of brain from premature fish.
Cells without lipofuscin pigments.
Nucleus (arrows) is centrally placed and has a moderate affinities with stain.
Ferric ferricyanide stain x 300.



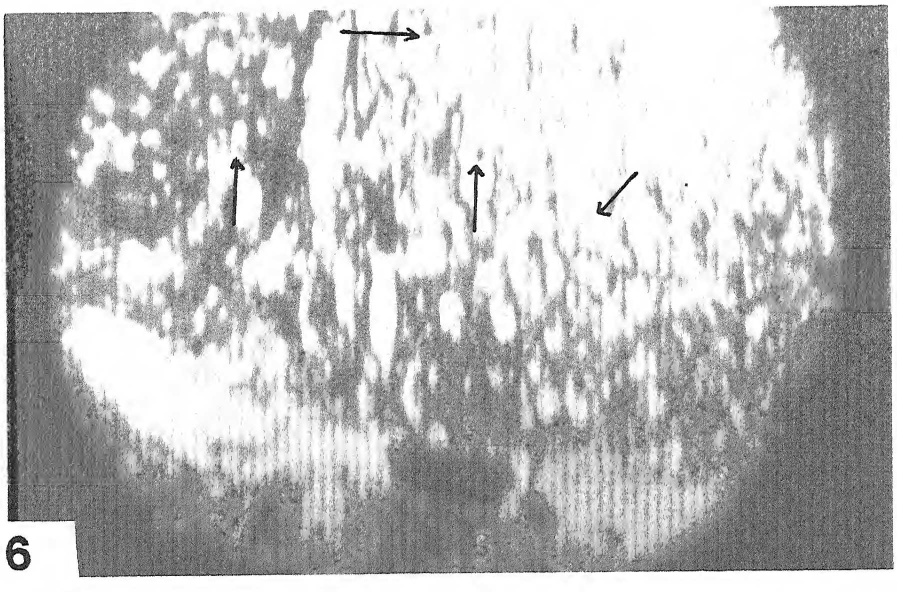
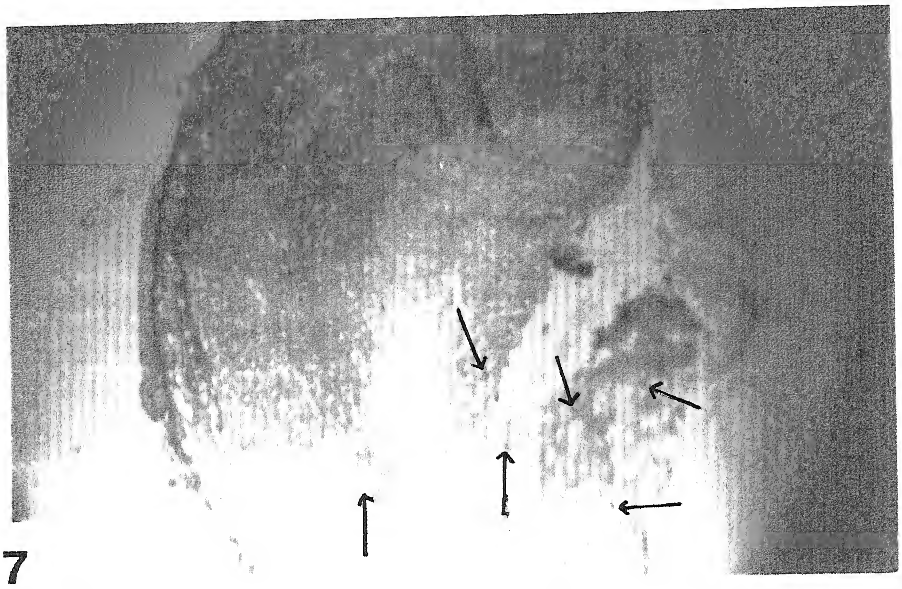


Fig. 7 : Section of brain from premature fish.
Cells without lipofuscin pigments.
Nile blue A stain x 300

Fig. 8 : Section of brain from premature fish.
Cells showing no pigments.
Nucleus (arrows) has great affinity with stain.
Carbol fuchsin stain x 450



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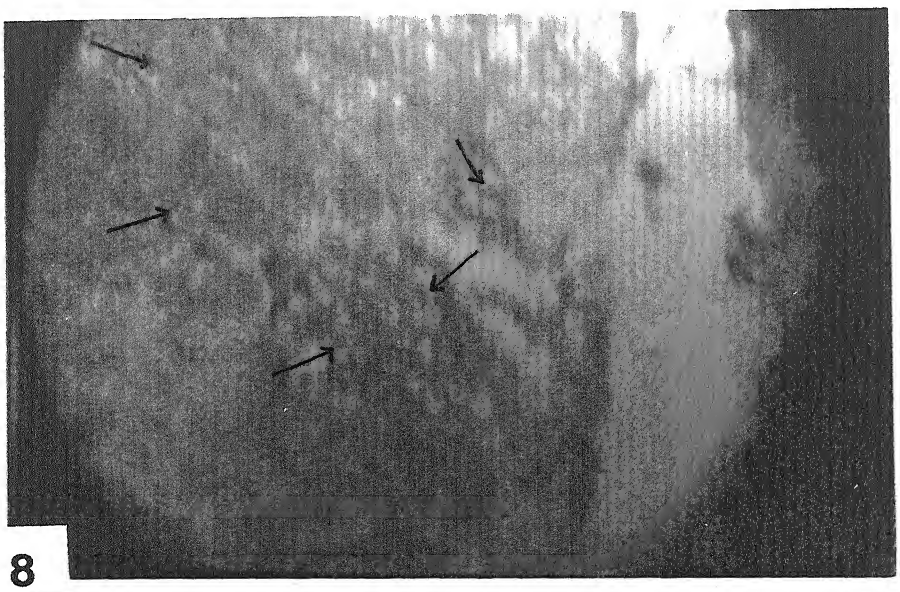

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AGE GROUP- 2

Mature Channa punctatus were included in the second age group. A comparative histological observations of heart and brain obtained from the (cultured and natural habitats) fishes of second age group at different age levels, related various age associated differences in the accumulation, morphology and distribution of lipofuscin pigment in the cytoplasm.

HEART,

Paraffin sections of the heart at different age levels from mature age group were stained and examined for accumulation, morphology and distribution of lipofuscin granules.

The observations revealed that the lipofuscin pigments were first appeared (Figs. 9,10,11) in the cells of heart as well as in brain cells of early mature fishes.

Lipofuscin pigment stained by the Nibe blue A, method appeared as dark blue green granules (Fig. 12) within the cytoplasm of cells. The pigment content of the cell was judge by the intensity of stain and the distribution of the pigment granules within the cells.

Observations of stained sections has revealed a moderate increase in the number of pigmented cells and pigment granules per cells. (Fig. 13).

Results revealed that some of the cells were found to contain lipofuscin pigment granules, where as other cells had no pigment in the same sections, and pigment accumulation within the cells and number of pigmented cells increased along with dark and blue green prominent pigment granules (Figs. 14,15).

Observations revealed that as the animal proceeds through the adult age there is a sharp decrease in the number of cells free from pigment granules and an increase in the number of cells containing pigment granules. Therefore, while the number of cells containing pigment increases with increasing age, the actual number of cells which show a great accumulation of pigments are relatively low. Counting of pigmented cells showed that approximately 40-60 % of cells were pigmented in this age group.

Table 1

Average mean percentage of pigmented cells in heart of mature fishes in natural and cultured (reared) habitat.

Age group	Habitat	Pigmented Cells/Volume	Cells Pigmented
		Mean ± S.E.	%
2	Natural Habitat	11 ± 0.28	55
	Cultured or Reared Habitat	9 ± 0.42	45

It has also been observed that the number of pigmented cells are more

in natural habitat of fishes than that of cultured (reared) habitat in laboratory. It is because due to environmental factors of lab. The results revealed that the accumulation of lipofuscin is also affected by the natural conditions of, the diet of the organism, light, humidity, water, air and lab environment respectively.

Morphological characteristics and distribution pattern of lipofuscin pigments were also examined in heart. During investigation it was observed that pigments appeared as rounded and homogeneous (Figs.16,17,18,19) in structure and increasing in size with age (Figs. 20,21,22,23,24) and lipofuscin granules were irregularly scattered throughout the cytoplasm of cells. At this stage two types of cells were noted i.e. cells containing homogeneous pigments and cells without pigments.

BRAIN,

Several paraffin sections of brain were also stained with Nile blue A, ferric ferricyanide and carbol fuchsin examined for first appearance, accumulation and distribution of lipofuscin pigment granules.

Observations revealed that the number of lipofuscin pigments and pigmented cells were remarkably less than those found in the heart.

Results revealed that at the beginning of this age group the cells of brain contained very few lipofuscin granules. The granules in the younger animals were first visible by means of the light microscope in tissues as a yellow pigment

in the form of tiny scattered particles distributed throughout the cytoplasm (Figs. 25,26,27) and as the animals increased in size the granules enlarged and tended to aggregate.The granules appeared to be larger in older age.

Observations revealed that the intracytoplasmic location of the pigment granules generally varied with age. In younger animals the granules were usually homogeneous, dispersed and located near the nucleus. and as the animal increased in size and advance in age, the pigment usually appeared dominantly and bigger in size. (Fig. 28)

Quantitation techniques showed that the volume of lipofuscin increased with the age of animals and this increasement took place in both natural and cultured habitats. The data indicates that the rate of lipofuscin accumulation in the cultured habitat is slower than that in natural habitat. The percentage of pigmented cells in brain were low and it was approximately 30-50 % in this age group.

Table 2

Average mean percentage of pigmented cells in brain of mature fishes in natural and cultured (reared) habitat.

Age group	Habitat	Pigmented Cells/Volume	Cells Pigmented
		Mean ± S.E.	%
2	Natural Habitat	9 ± 0.51	45
	Cultured or Reared Habitat	8 ± 0.42	40

A comparative detailed account of morphological characteristics and distribution pattern of lipofuscin pigment in different tissues (heart and brain) of second age group shown in table No 3. In the heart and brain one common characteristics of lipofuscin pigment was observed that the lipofuscin pigment granules were homogeneous. and in both the tissues the pigment granules were irregularly distributed throughout the cytoplasm.

power of microscope to show the chemical nature of lipofuscin pigment granules.

It was clearly observed that one stain had strong affinities with the pigment granules on the other hand same stain had mild or moderate affinities with pigment of the same tissues at different age levels.

In the second age group lipofuscin pigment granules of heart had strong affinities with Nile blue A, stain where as affinities were moderate with ferric ferricyanide and carbol fuchsin. The staining behaviour was quite different in brain as cells has strong affinities with Nile blue A, moderate with ferric ferri cyanide and mild with carbol fuchsin.

Table 4

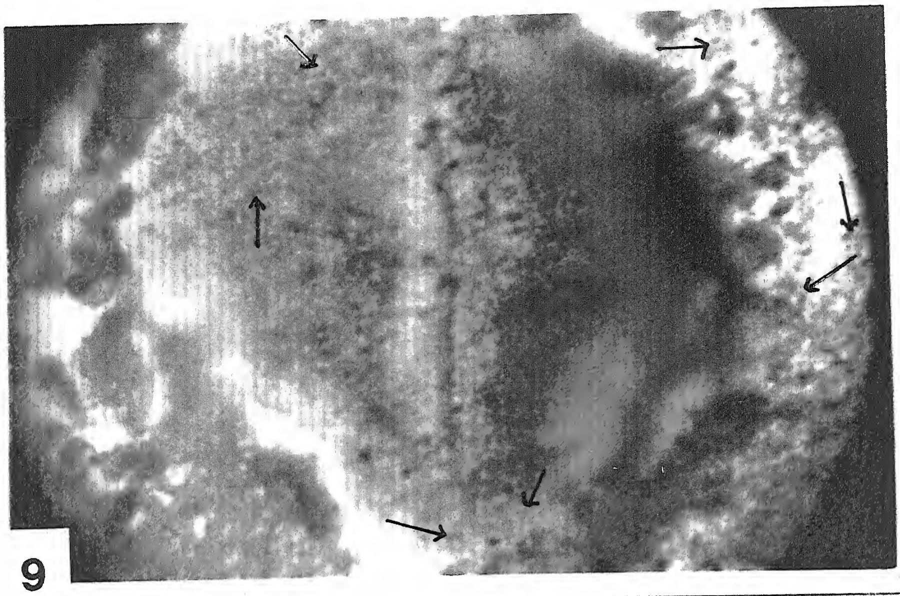
Histochemical nature of lipofuscin pigment in different tissues in second age group.

Age group	Tissues	Stains		
		Nile blue A	Ferric Ferri-cyanide	Carbol Fuchsin
2	Heart	+ + +	+ +	+ +
	Brain	+ + +	+ +	+

+ + + STRONGLY POSITIVE, + + MODERATELY POSITIVE, + MILD POSITIVE

Fig. 9 : Section of heart from early mature fish. Cells showing first or early appearance of lipofuscin pigment granules , (arrows). Carbol fuchsin stain x 450.

Fig. 10 : Section of brain from early mature fish. Cells showing first appearance of lipofuscin pigment granules (arrows). Nile blue A stain x 450



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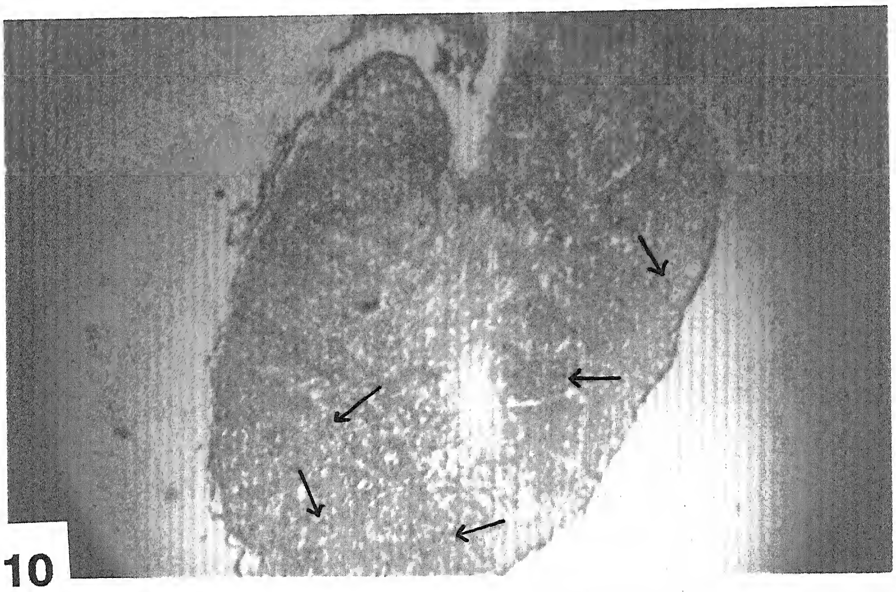

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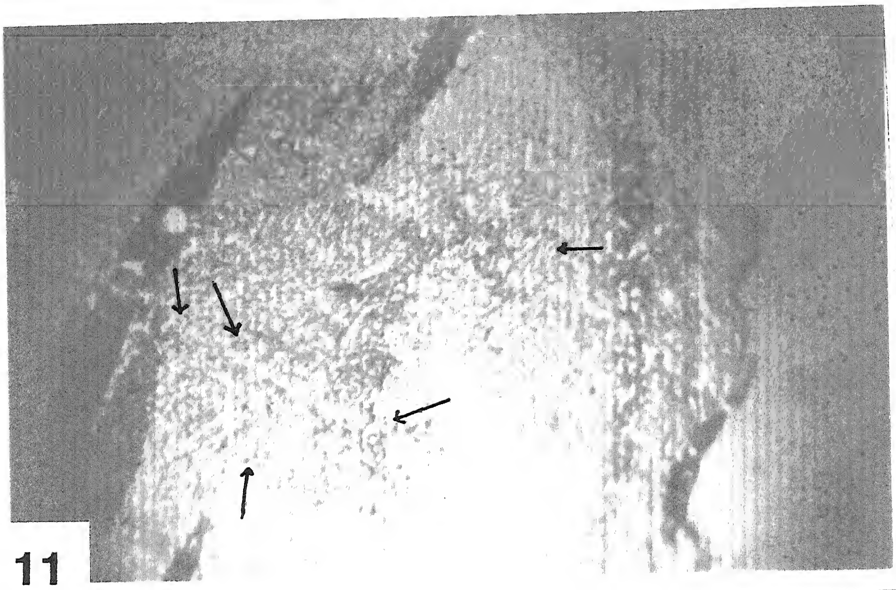
Fig. 11 : Section of brain from early mature fish.
Cells showing first appearance of lipofuscin pigment granules (arrows).
Ferric ferricyanide stain x 300

Fig. 12 : Section of heart from mature fish.
Cells showing early appearance of lipofuscin pigment granules (arrows).
Pigment appeared as tiny dot like and blue green in colour.
Nile blue A stain x 450.



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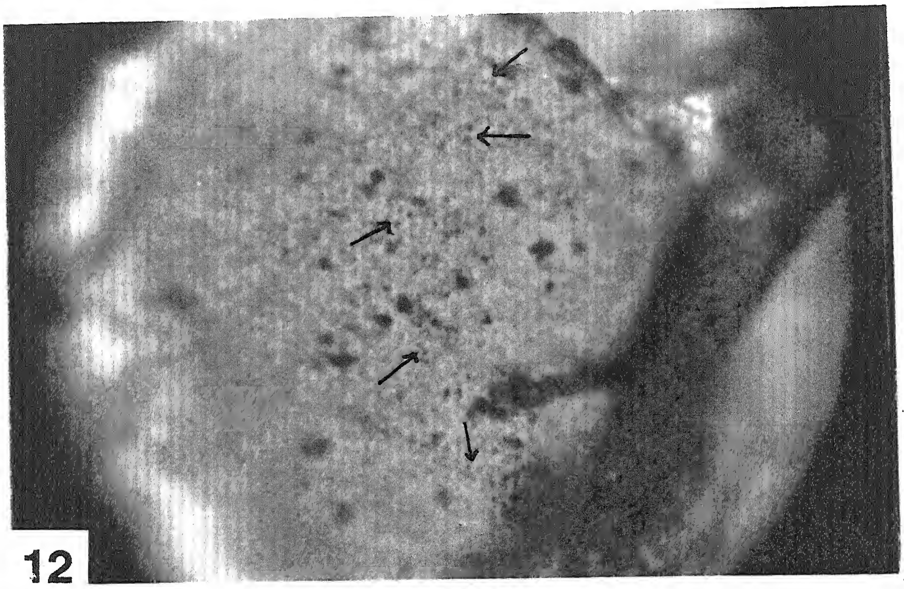

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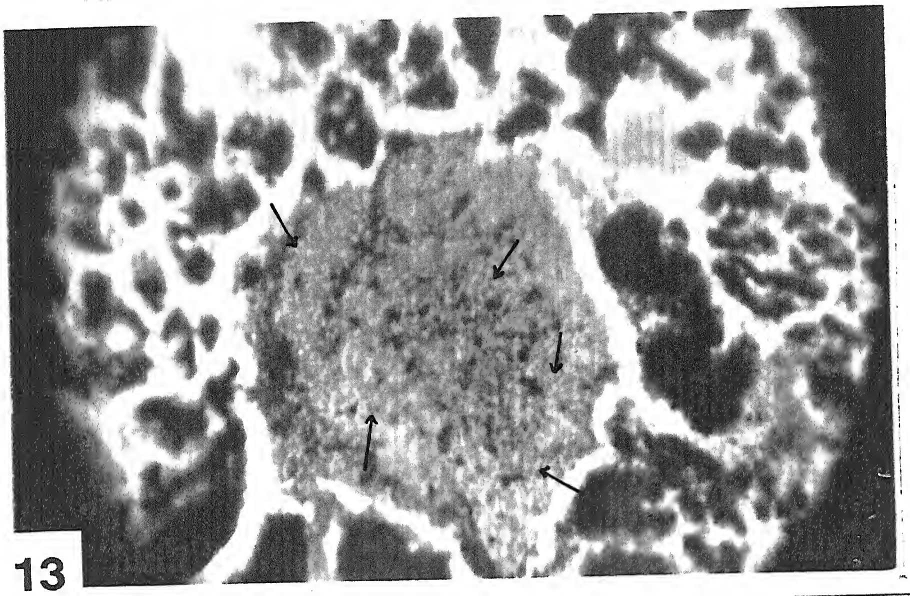
Fig. 13 : Section of heart from mature fish.
Cells showing increased accumulation of lipofuscin pigment granules (arrows).
Ferric ferricyanide stain x 450.

Fig. 14 : Section of heart from mature fish.
Cells showing increased accumulation of dark blue green prominent pigment granules (arrows).
Carbol fuchsin stain x 450.



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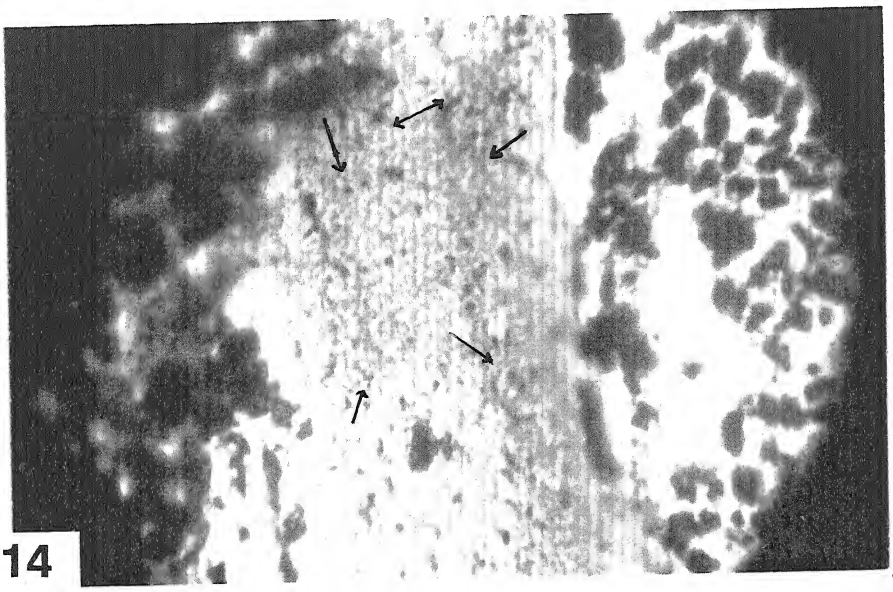

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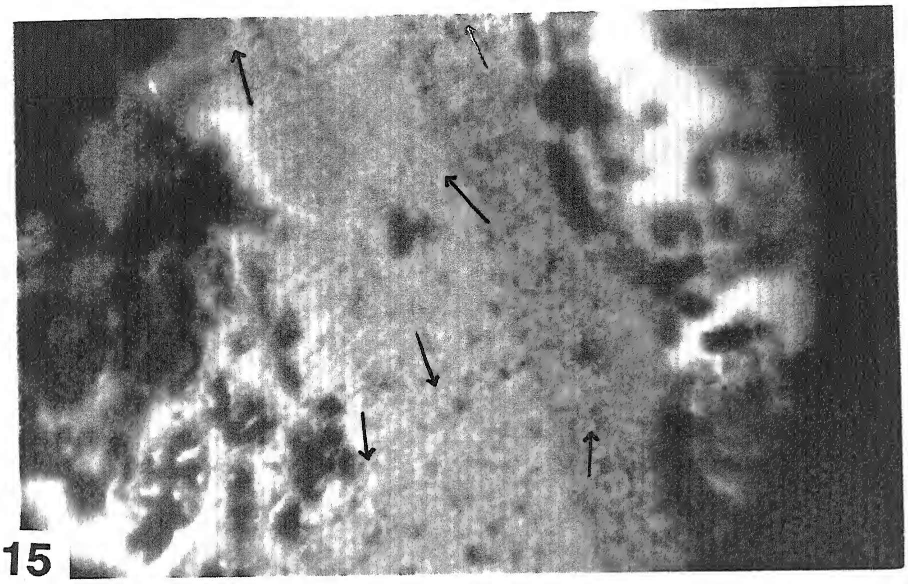
Fig. 15 : Section of heart from mature fish.
Cells showing increased accumulation of lipofuscin pigment with dark blue green prominent pigments (arrows).
Nile blue A stain x 450.

Fig. 16 : Section of heart from mature fish.
Cells showing moderate rounded and homogeneous pigment granules (arrows) scattered throughout the cytoplasm.
Nile blue A stain x 300.



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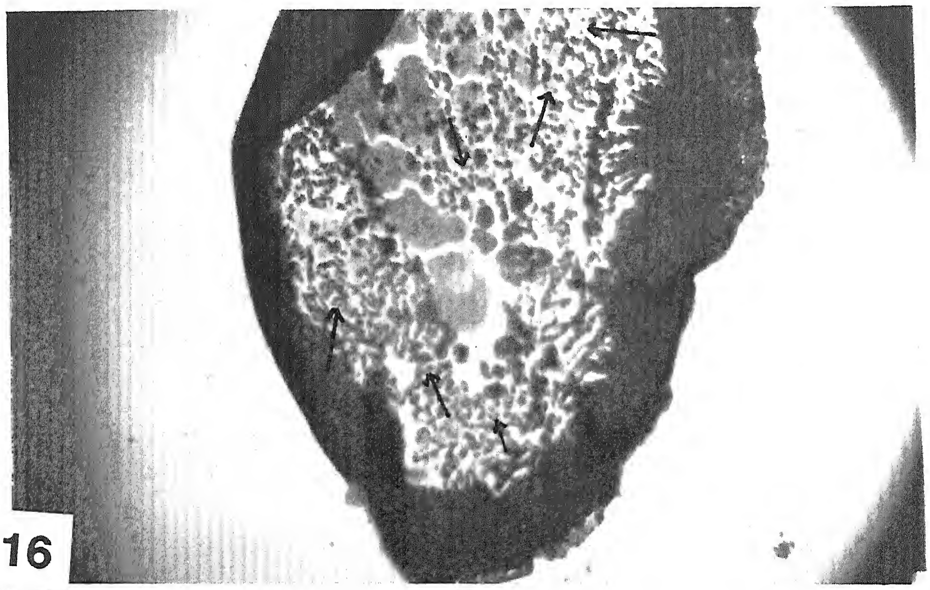

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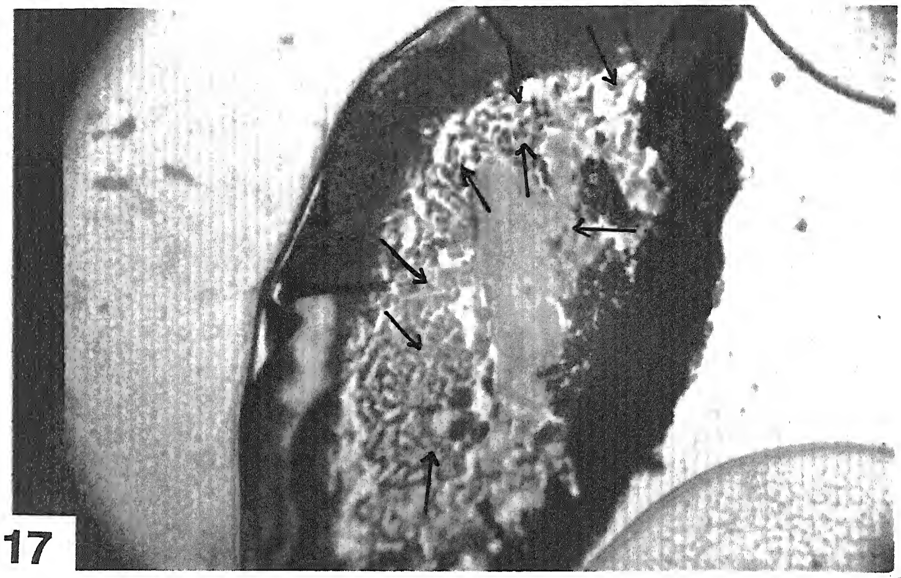
Fig. 17 : Section of heart f om mature fish.
Cells showing rounded and homogeneous pigment granules (arrows) distributed throughout the cytoplasm.
Ferric ferricyanide stain x 300.

Fig. 18 : Section of heart from mature fish.
Cells showing rounded and homogeneous pigment granules (arrows) distributed throughout the cytoplasm.
Ferric ferricyanide stain x 450.



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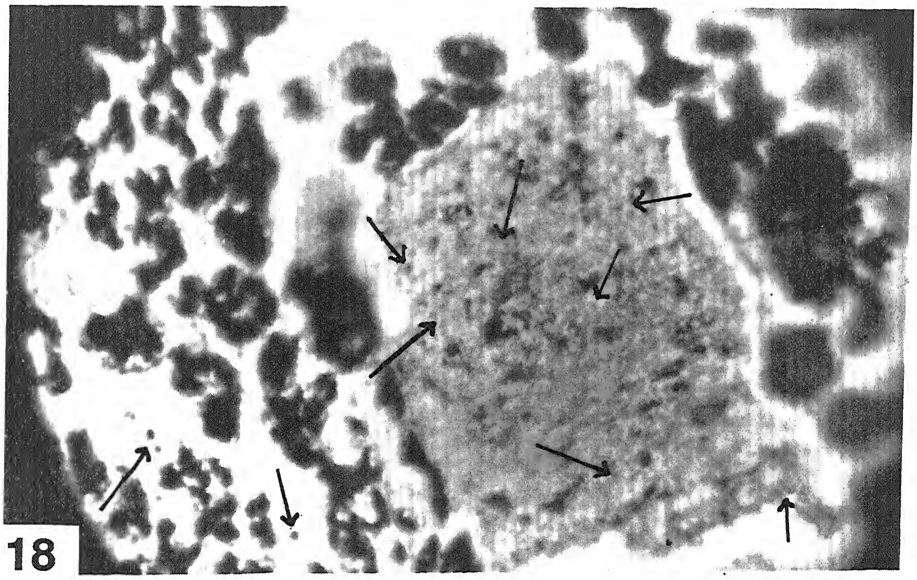

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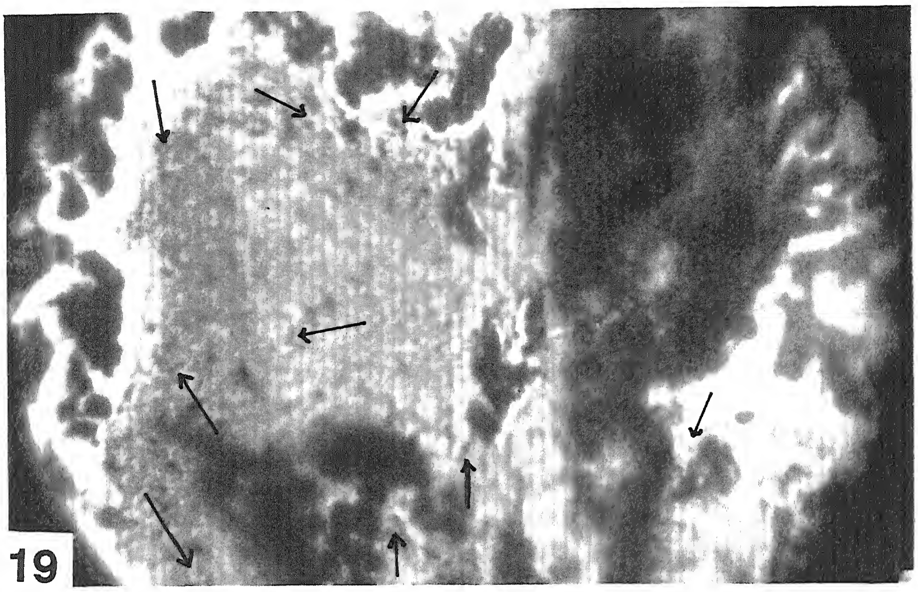
Fig. 19 : Section of heart from mature fish.
Cells showing moderate, rounded and homogeneous and dense pigment granules (arrows) irregularly distributed throughout the cytoplasm.
Nile blue A x 450.

Fig. 20 : Section of heart from late mature fish.
Cells showing dense and large scattered pigment granules (arrows).
Ferric ferricyanide stain x 450.



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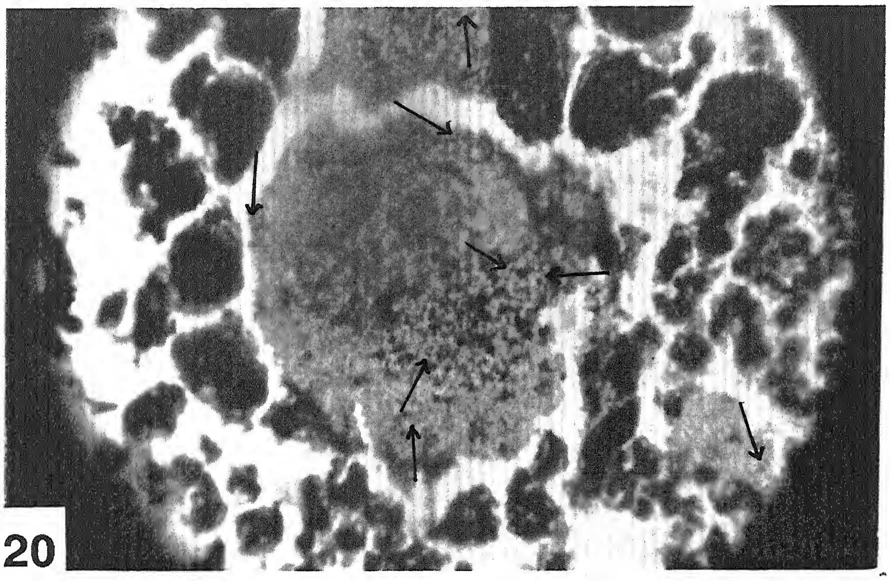

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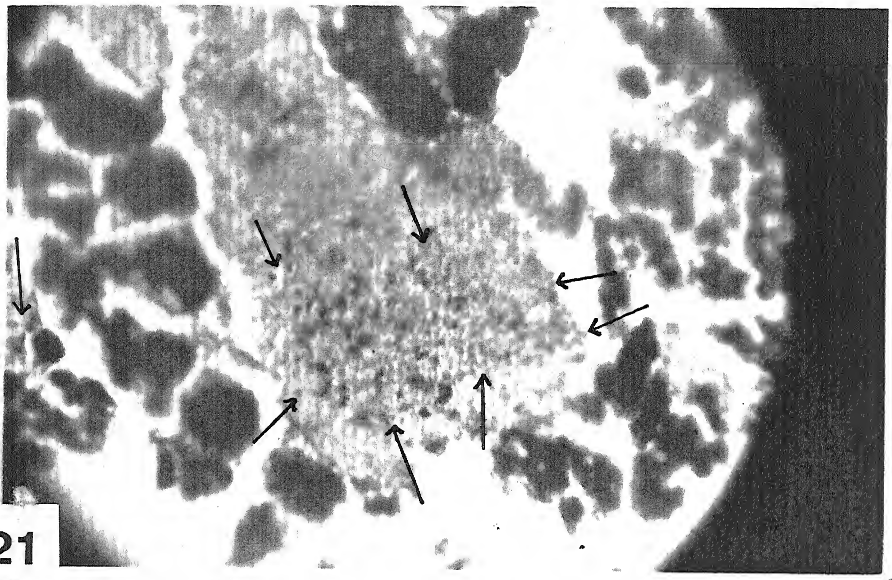
Fig. 21 : Section of heart from late mature fish. Cells showing increased accumulation of irregularly distributed lipofuscin pigment granules (arrows) .
Nile blue A stain x 450.

Fig. 22 : Section of heart from late mature fish. Cells showing increased accumulation of lipofuscin pigment granules (arrows).
Ferric Ferricyanide stain x 300



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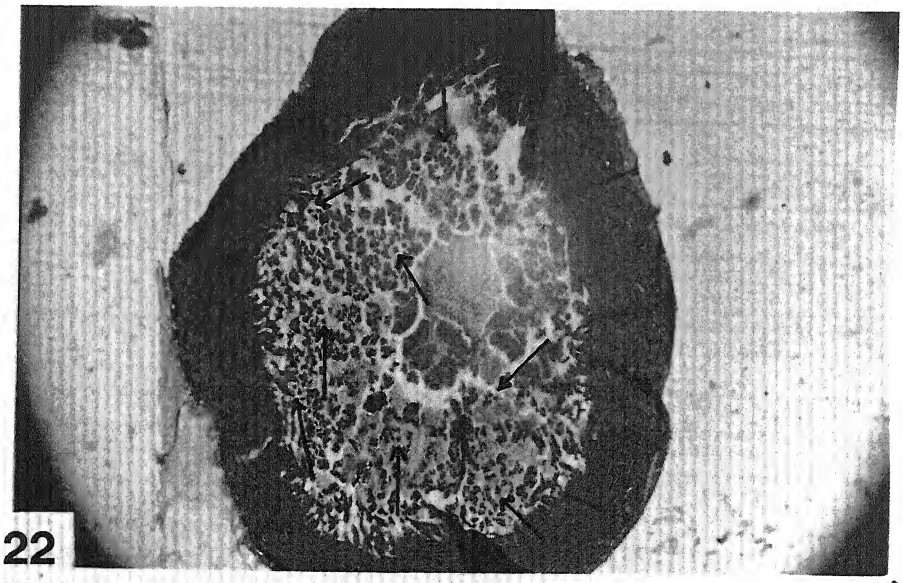

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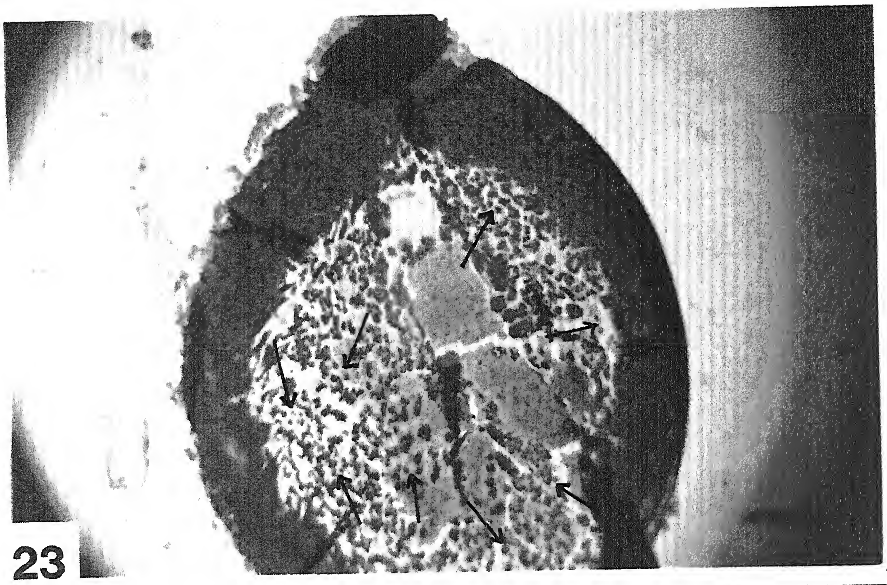
Fig. 23 : Section of heart from late mature fish.
Cells showing dense and increased accumulation of lipofuscin pigment granules (arrows) within the cytoplasm.
Nile blue A stain x 300

Fig. 24 : Section of heart from late mature fish.
Cells showing dense and increased distribution of lipofuscin pigment granules (arrows).
Carbol fuchsin stain x 300



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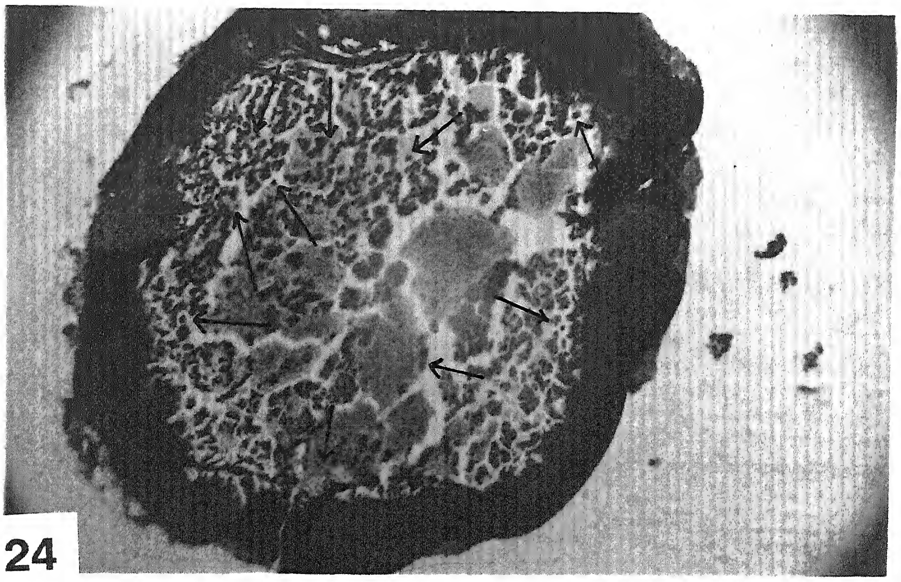

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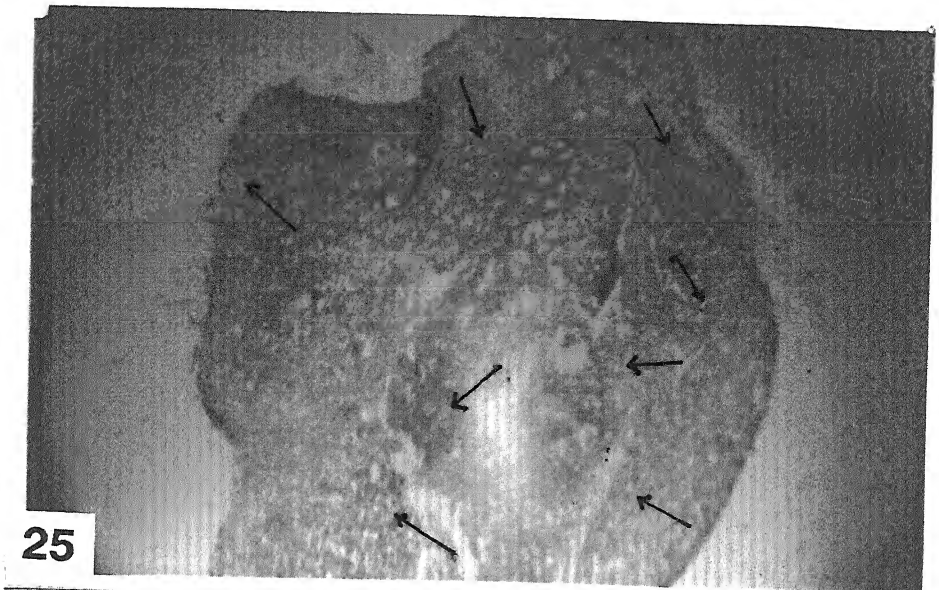
Fig. 25 : Section of brain from mature fish.
Cells showing tiny small lipofuscin pigment granules (arrows) scattered throughout the cytoplasm.
Nile blue A stain x 300.

Fig. 26 : Section of brain from mature fish.
Cells showing small rounded and homogeneous lipofuscin pigment granules (arrows).
Ferric ferricyanide stain x 300.



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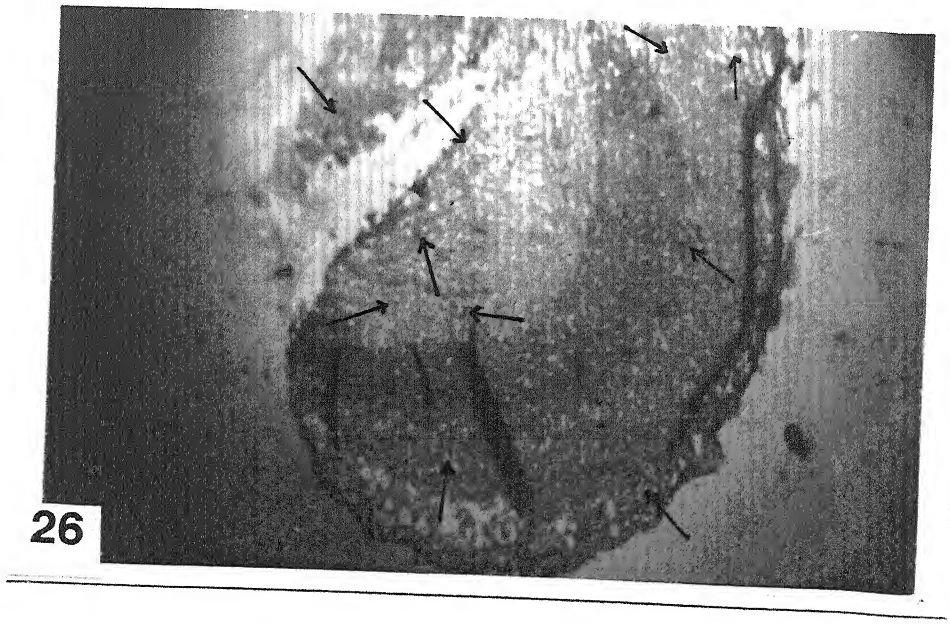

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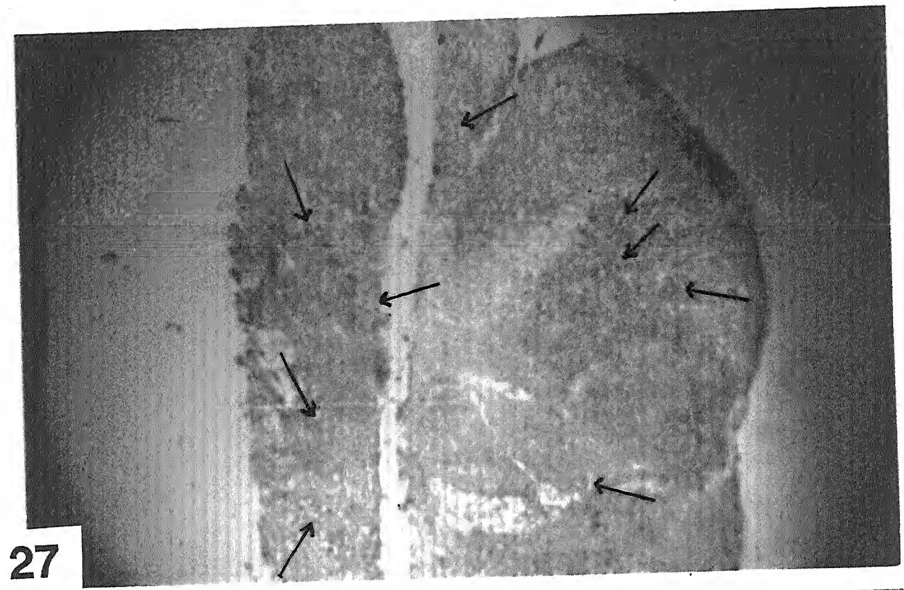
Fig. 27 : Section of brain from mature fish.
Cells showing small, rounded and homogeneous lipofuscin pigment granules (arrows) distributed throughout the cytoplasm.
Carbol fuchsin stain x 300.

Fig. 28 : Section of brain from mature fish.
Cells showing large and dominant lipofuscin pigment granules (arrows).
Nile blue A stain x 300.



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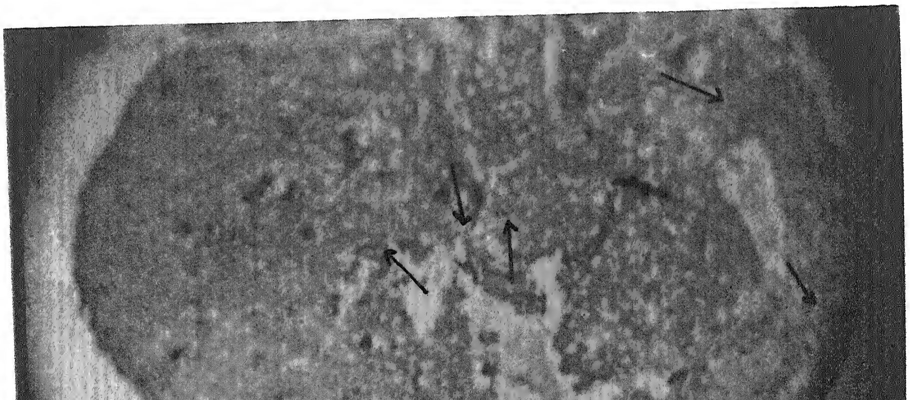

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AGE GROUP -3.

The third age group includes the postmature Channa punctatus for a detailed study of lipofuscin pigments. Though the previous age groups showed that lipofuscin pigment granules increases gradually and lie scattered in the cytoplasm but the observations of the third age group revealed that pigment granules have a tendency to aggregate in groups. Moreover several aggregated groups of pigment granules were observed particularly in senescent cells of heart and brain. Nevertheless the number of pigmented cells also steadily increased with age. Different types of accumulation and distribution of pigment granules were also noted in the present investigation.

HEART.

The paraffin sections of the heart were stained and examined for lipofuscin pigment granules. A comparative histological examinations of the heart cells revealed various age associated differences in number and distribution of dense pigment granules in the cytoplasm. Variable size and amount of lipofuscin pigments were also observed in the heart cells at different age levels, and heart cells showed a clear aggregation of pigment granules. Some of the pigment granules which were observed in aggregated forms appeared as complete masses in older age. Such changes show an interesting characteristics of fusion among the pigment granules, however these aggregated pigment granules were more heterogeneous and more irregular,(Figs.29,30). Observations revealed that

as animals proceed gradually through old age there is an increase in the number of cells containing lipofuscin pigments and a decrease in the number of cells free from pigments. Counting of the pigmented cells showed that approximately 60-85 % cells were pigmented in this age group.

Table 5

Average mean percentage of pigmented cells in heart in different age groups.

Age group	Tissues	Pigmented Cells/Volume	Cells Pigmented
		Mean ± S.E.	%
Premature	Heart	-	-
Mature	Heart	10 ± 0.56	50
Post Mature	Heart	15 ± 0.42	75

Morphological characteristics and distribution pattern of lipofuscin pigments were also examined. Observations revealed that granules in the younger animals were just visible even by means of the light microscope. As the animals increased in size the granules enlarged and tended to form clusters and appeared larger in size.

Results obtained from the observations revealed that a greater number of pigmented cells contained heterogeneous and clumped or clustered pigments instead of dispersed granules in the cytoplasm and the clumped granules

generally scattered in the cytoplasm of cells. Lipofuscin pigment granules were so abundant that they generally occupied the entire cytoplasmic area of cells, however few pigments were deposited at the periphery of the cells (Figs. 31,32,33,34). Heterogeneous pigment granules within the cells were observed only in this third age group. It was also noted that the size, complexity and intra cellular distribution of the pigmented granules were variable within the cells at different age levels. All the four categories of cells were found in the heart during investigation of third age group.

Table 6

Morphological characteristics and distribution of lipofuscin pigments in heart in different age groups.

Age group	Tissues	Morphological characteristics of lipofuscin pigments	Distribution of lipofuscin pigments
Premature	Heart	No pigments	No pigments
Mature	Heart	Homogeneous, appeared tiny to larger in size	Irregularly scattered throughout the cytoplasm
Post mature	Heart	Heterogeneous, majority of cells contain clumped pigments.	Pigments generally scattered throughout the cytoplasm, few located at the periphery.

BRAIN,

Several paraffin sections of brain were also stained with Nile blue A, ferric ferricyanide and carbol fuchsin and examined for accumulation structural morphology and distribution pattern of lipofuscin pigment granules. The most striking finding was the heterogeneous lipofuscin pigment granules were observed in masses in some cells of brain.

Results revealed that pigments were present in neurones of most areas of the brain in older age, although scattered ones could be seen in early ages. The pigmentation was rather slow (Figs. 35,36) up to a certain age level after which it increased considerably. There was a heavy deposition of lipofuscin (Figs. 37,38) in older age and these occupied a considerable area within the cytoplasm of the cells. The pigmentation usually started as granules diffusely distributed in the cytoplasm. As the pigmentation progressed these appeared in to form of clusters around the nucleus. This is a common type of distribution found in many neurones in various cells in third age group.

Observations revealed that dense bodies were more heterogeneous and more irregular in outline in the older animals and there was a tendency for these structures to aggregate in to groups (clusters) throughout the cytoplasm. Results revealed that pigmented cells increased in number with increasing age of fish. Counting of pigmented cells showed that approximately 50-75 % of cells

were pigmented in this age group and the percentage of pigmentation is higher than second age group.

Table 7

Average mean percentage of pigmented cells in brain in different age groups.

Age group	Tissues	Pigmented Cells/Volume Mean ± S.E.	Cells Pigmented %
Premature	Brain	-	-
Mature	Brain	9 ± 0.73	45
Post Mature	Brain	14 ± 0.32	70

A comparative account of morphological characteristics and distribution of lipofuscin pigment granules in brain in different age group is given in table 8.

Table 8

Detailed account of morphological characteristics and distribution of lipofuscin in brain in different age groups.

Age group	Tissues	Morphological characteristics of lipofuscin pigments	Distribution of lipofuscin pigments
Premature	Brain	No pigments	No pigments
Mature	Brain	Homogeneous, small granules becomes dense	Irregularly scattered throughout the cytoplasm
Post mature	Brain	Heterogeneous, granules generally aggregated in masses	Uniformly distributed throughout the cytoplasm, but few deposited at the peri--phery of cells.

Results obtained from the observations revealed that lipofuscin pigments were variable in different tissues in different age groups. It was noted that the accumulation and complexity of lipofuscin granules increases with age.

Examinations of histological sections suggests that each pigmented cell may contain scattered/aggregated granules, but majority of cells contain heterogeneous pigments. Lipofuscin granules in brain cells were comparatively smaller in size than the pigments of the heart. The low and less pigmentation were also noted in brain cells as compared to those of heart at different age levels

in third age group.

Table 9

Morphological characteristics and distribution of lipofuscin in different tissues in third age group.

Age group	Tissues	Morphological characteristics of lipofuscin pigments	Distribution of lipofuscin pigments
3	Heart	Heterogeneous, majority of cells contain clumped pigments.	Pigments scattered throughout the cytoplasm and few located at the periphery.
	Brain	Heterogeneous, granules generally aggregated in masses.	Uniformly distributed throughout the cytoplasm and few located at the periphery.

Results obtained from the observations revealed that morphological characteristics and distribution pattern of lipofuscin pigments granules in heart and brain were quite different in this age group. One common characteristic was observed in this age group i.e. heterogeneous appearance of lipofuscin pigment within the cell.

HISTOCHEMICAL OBSERVATIONS

In third age group, maximum accumulation and similar morphological characteristics of lipofuscin pigments were observed in heart and brain. The lipofuscin granules of heart had strong affinities with Nile blue A and ferric

ferricyanide while moderately stains with carbol fuchsin. The lipofuscin granules of brain cells also reacted similarly. Table 10 shows that pigment granules in heart and brain reacted strongly and moderately with different stains i.e. Nile blue A, ferric ferricyanide and carbol fuchsin.

Table 10

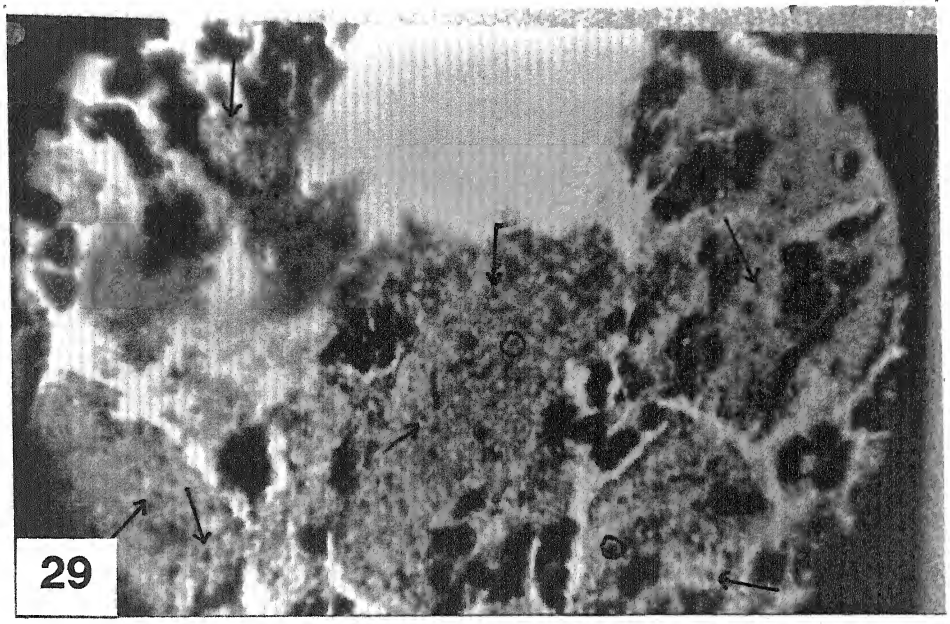
Showing staining behavior of lipofuscin pigment granules in different tissue in third age group.

Age group	Tissues	Stains		
		Nile blue A	Ferric Ferri-cyanide	Carbol Fuchsin
3	Heart	+ + +	+ + +	+ +
	Brain	+ + +	+ + +	+ +

+ + + STRONGLY POSITIVE, + + MODERATELY POSITIVE, + MILD POSITIVE

Fig. 29 : Section of heart from postmature fish.
Cells showing dense, clumped, heterogeneous lipofuscin pigment granules (arrows) irregularly distributed throughout the cytoplasm.
Ferric ferricyanide stain x 450.

Fig. 30 : Section of heart from postmature fish.
Cells showing dense clustered lipofuscin pigments (arrows) with heavy deposition.
Nile blue A stain x 450.



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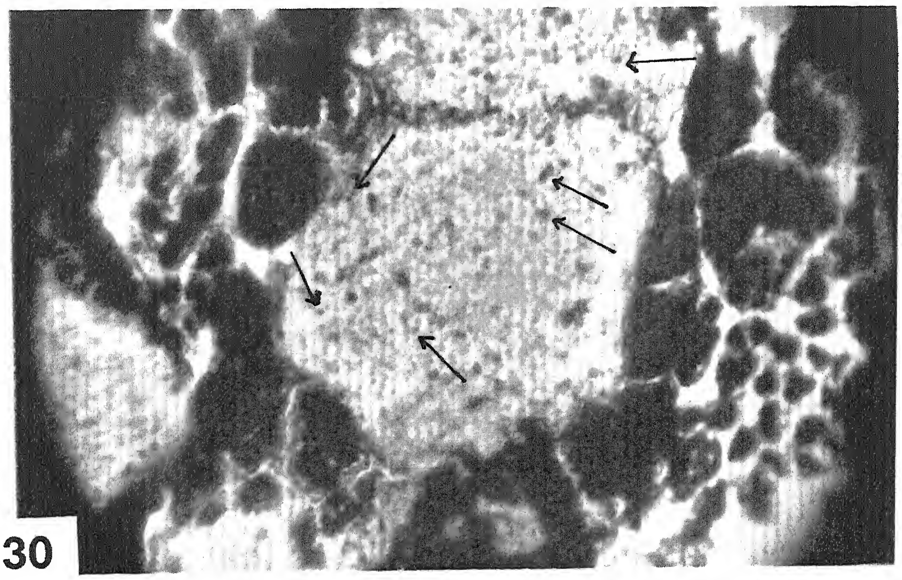

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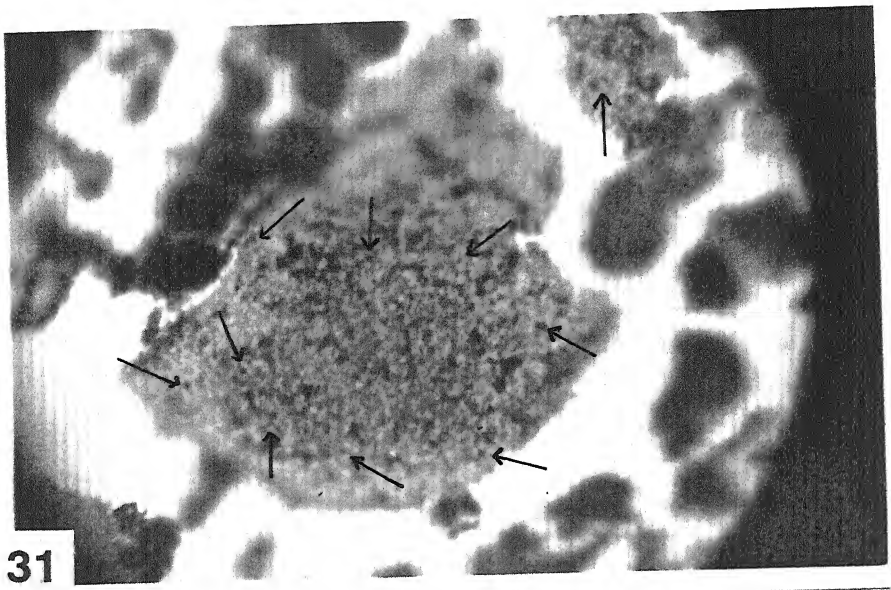
Fig. 31 : Section of heart from postmature fish.
Cells showing clumped heterogeneous lipofuscin pigment granules (arrows) few located at the periphery.
Carbol fuchsin stain x 450.

Fig. 32 : Section of heart from postmature fish.
Cells showing dense, heterogeneous lipofuscin pigment granules (arrows) scattered throughout the cytoplasm.
Carbol fuchsin stain x 300.



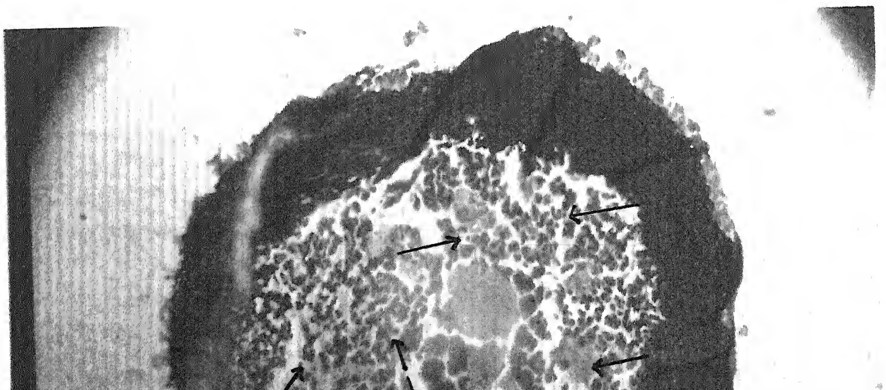
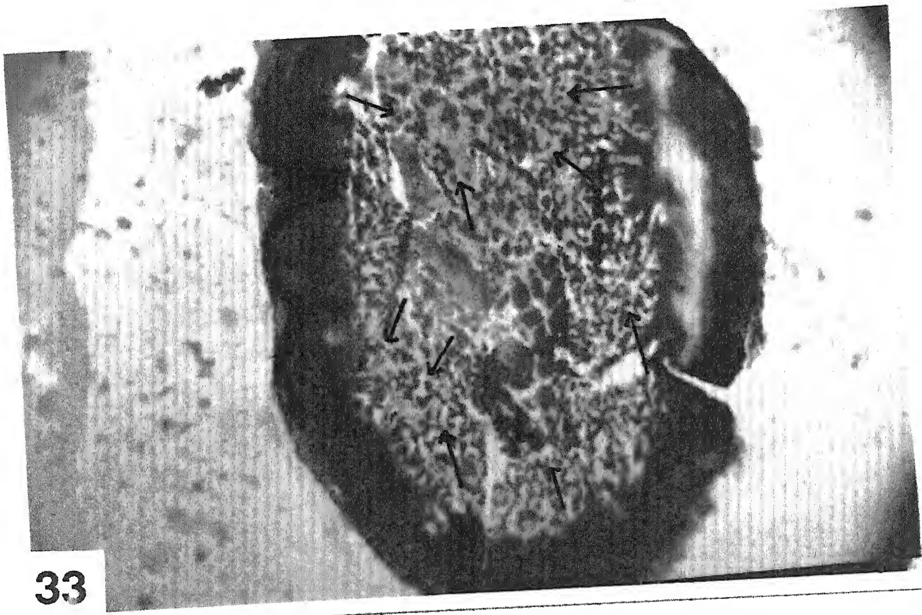


Fig. 33 : Section of heart from postmature fish.
Cells showing heavy deposition of clumped, heterogeneous lipofuscin pigment granules (arrows) throughout the cytoplasm.
Nile blue A stain x 300.

Fig. 34 : Section of brain from postmature fish.
Cells showing heavy deposition of clumped, heterogeneous lipofuscin pigment granules (arrows) throughout the cytoplasm.
Ferric ferricyanide stain x 300.



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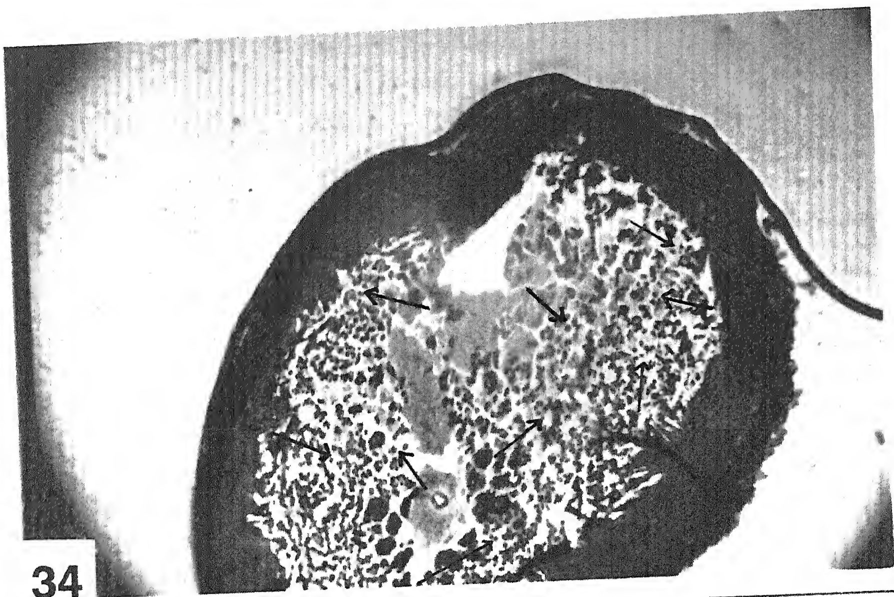

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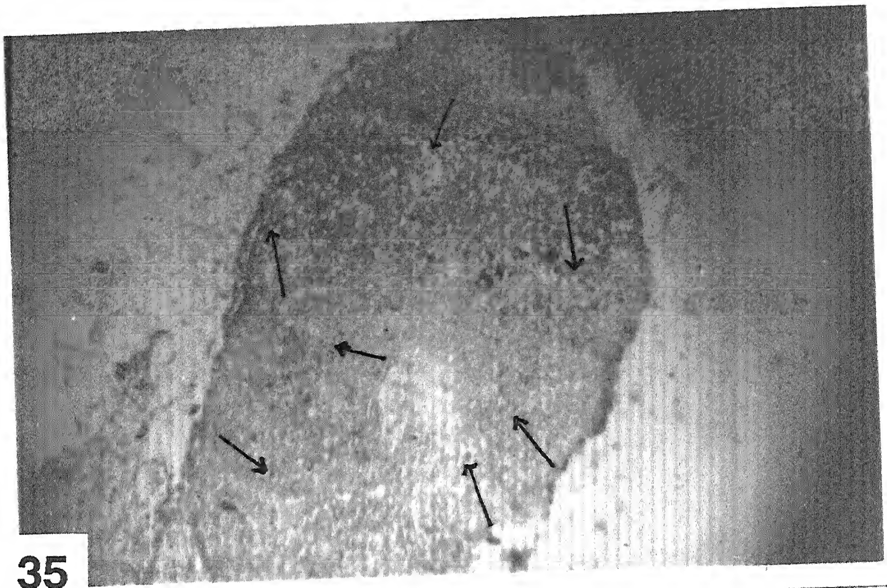
Fig. 35 : Section of brain from postmature fish.
Cells showing aggregated heterogeneous pigments (arrows) irregularly scattered throughout the cytoplasm.
Carbol fuchsin stain x 300.

Fig. 36 : Section of brain from postmature fish.
Cells showing dense, clumped, heterogeneous lipofuscin pigment granules (arrows) distributed throughout the cytoplasm.
Nile blue A stain x 300.



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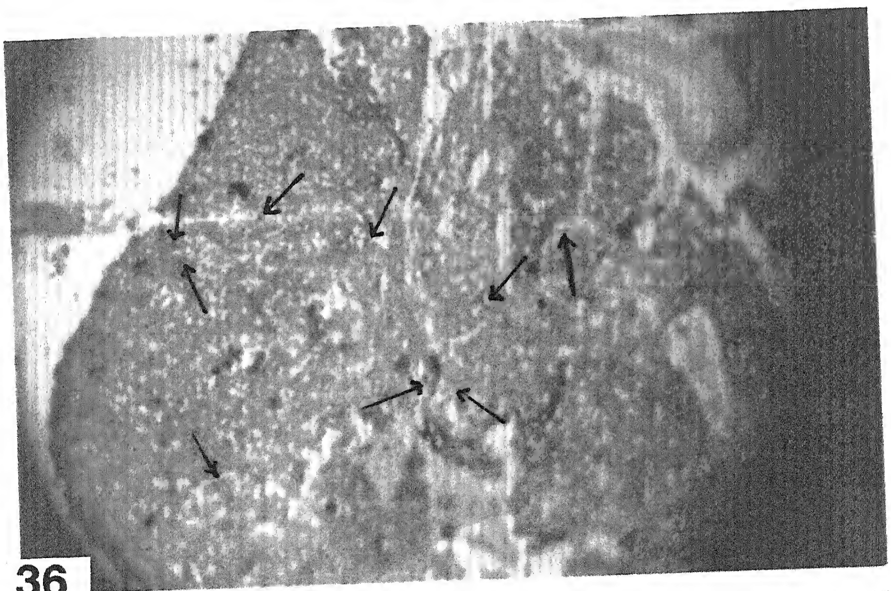

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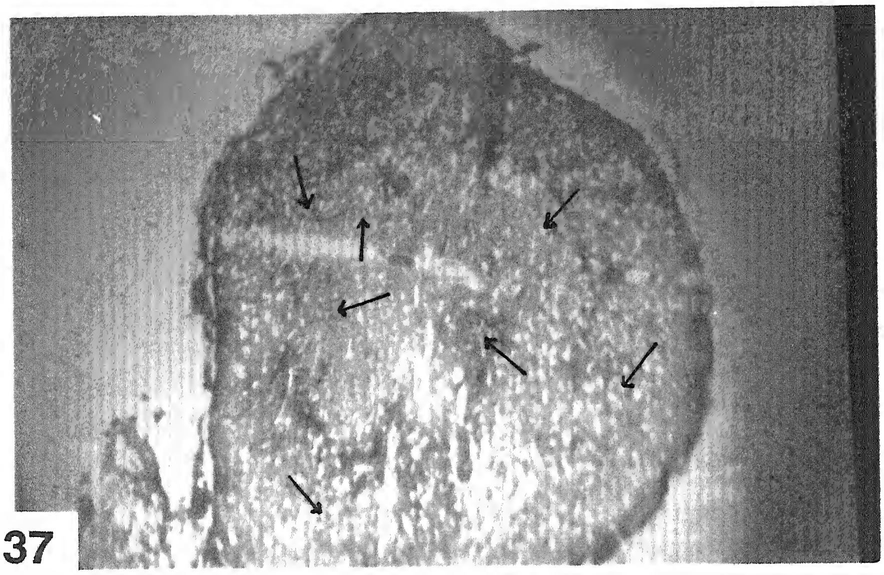
Fig. 37 : Section of brain from postmature fish.
Cells showing increased accumulation of heterogeneous lipofuscin pigment granules (arrows)
Ferric ferricyanide stain x 300

Fig. 38 : Section of brain from postmature fish.
Cells showing heavy deposition of heterogeneous lipofuscin pigment granules (arrows)
Nile blue A stain x 300.



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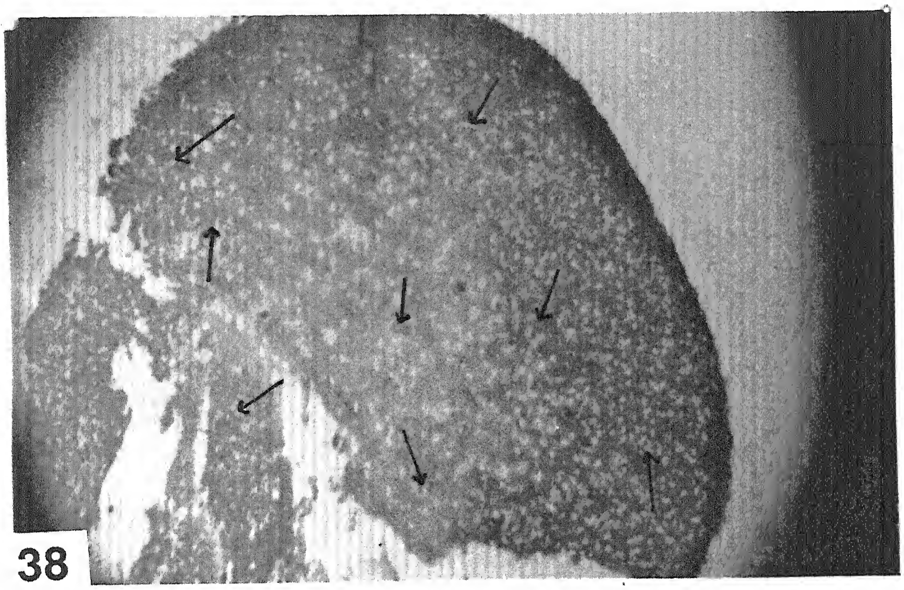

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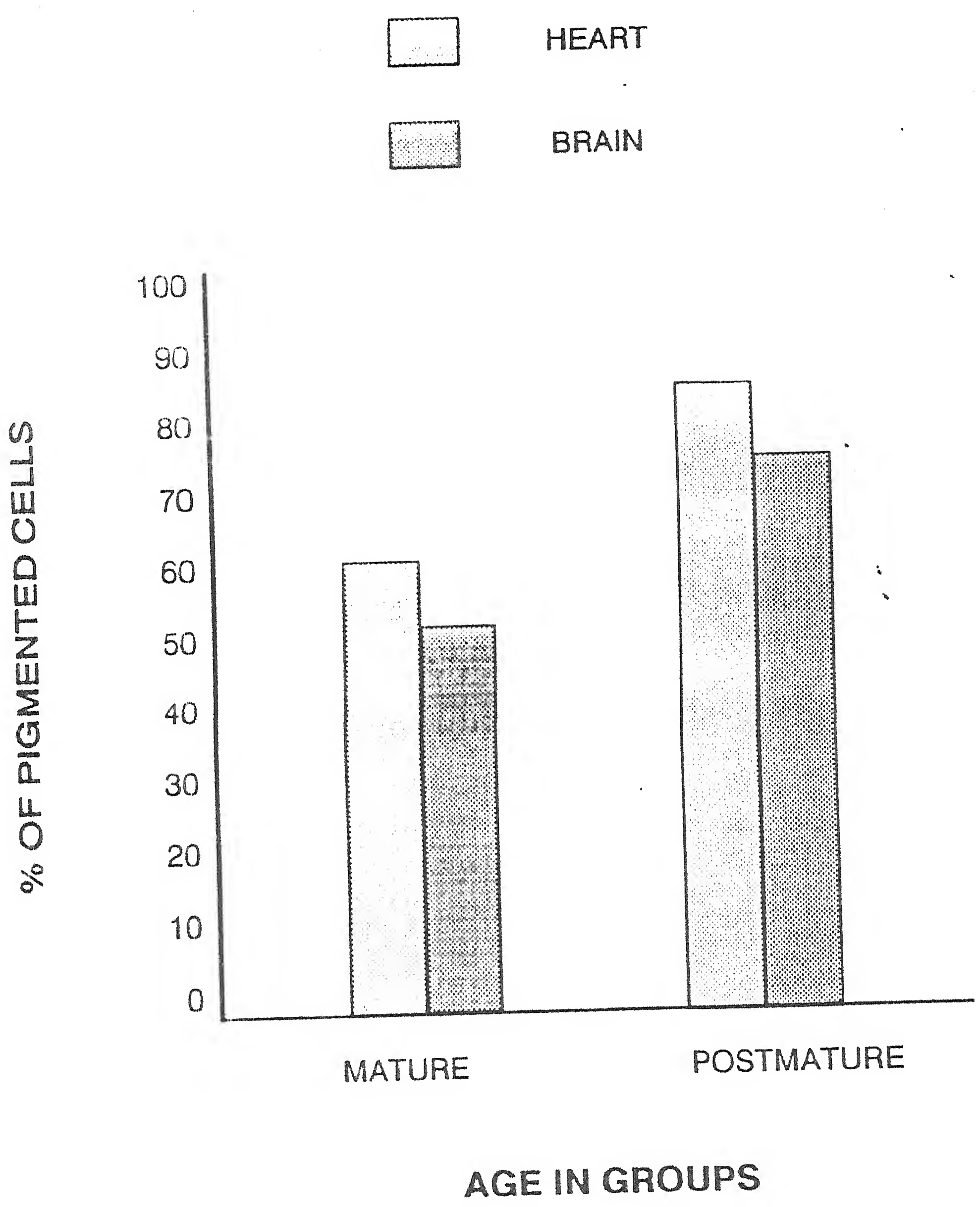

Fig. - Maximum Percentage of Pigmented Cells in Heart and Brain of Channa punctatus in diffrent age groups.

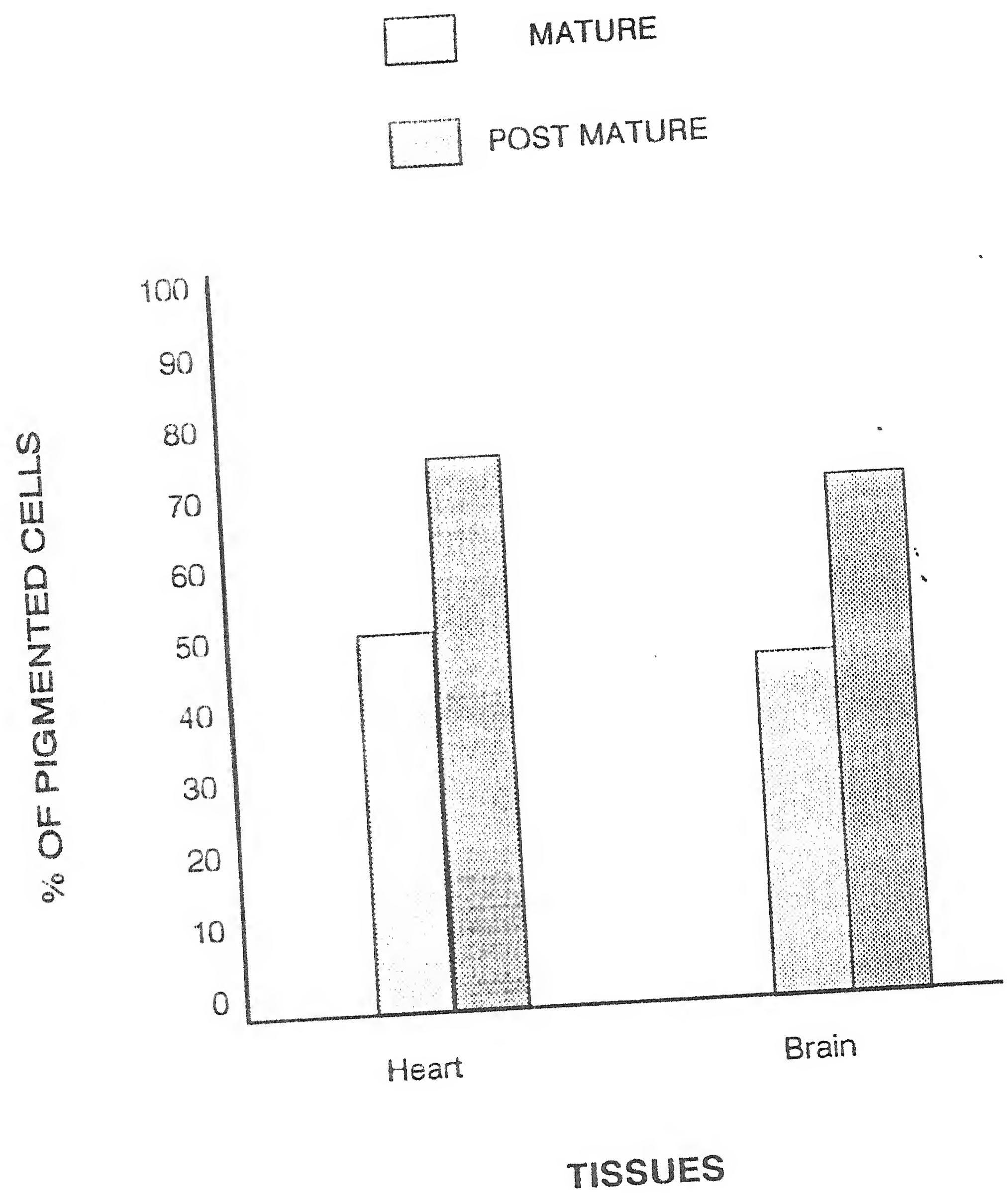

Fig. - Average Mean Percentage of Pigmented Cells in Heart and Brain of <u>Channa punctatus</u> in diffrent age groups.

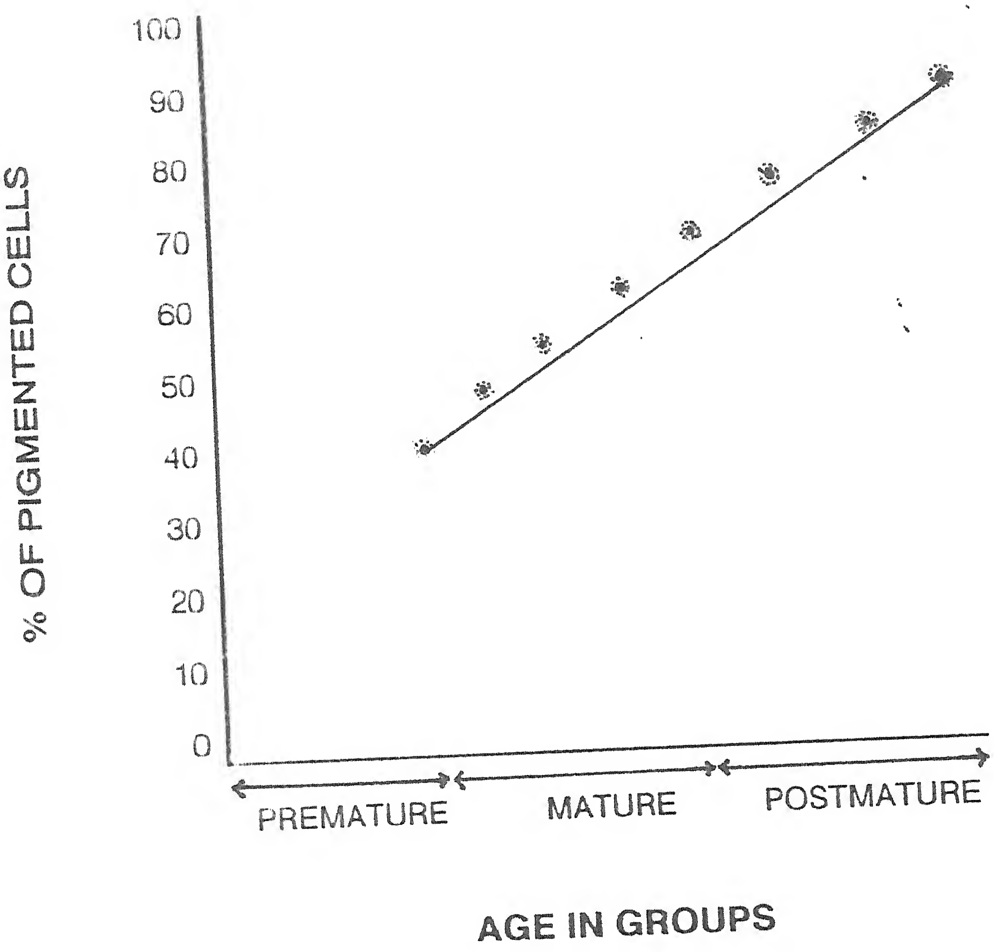

Fig. - Percentage of Pigmented Cells in Heart vs age for <u>Channa punctatus</u>

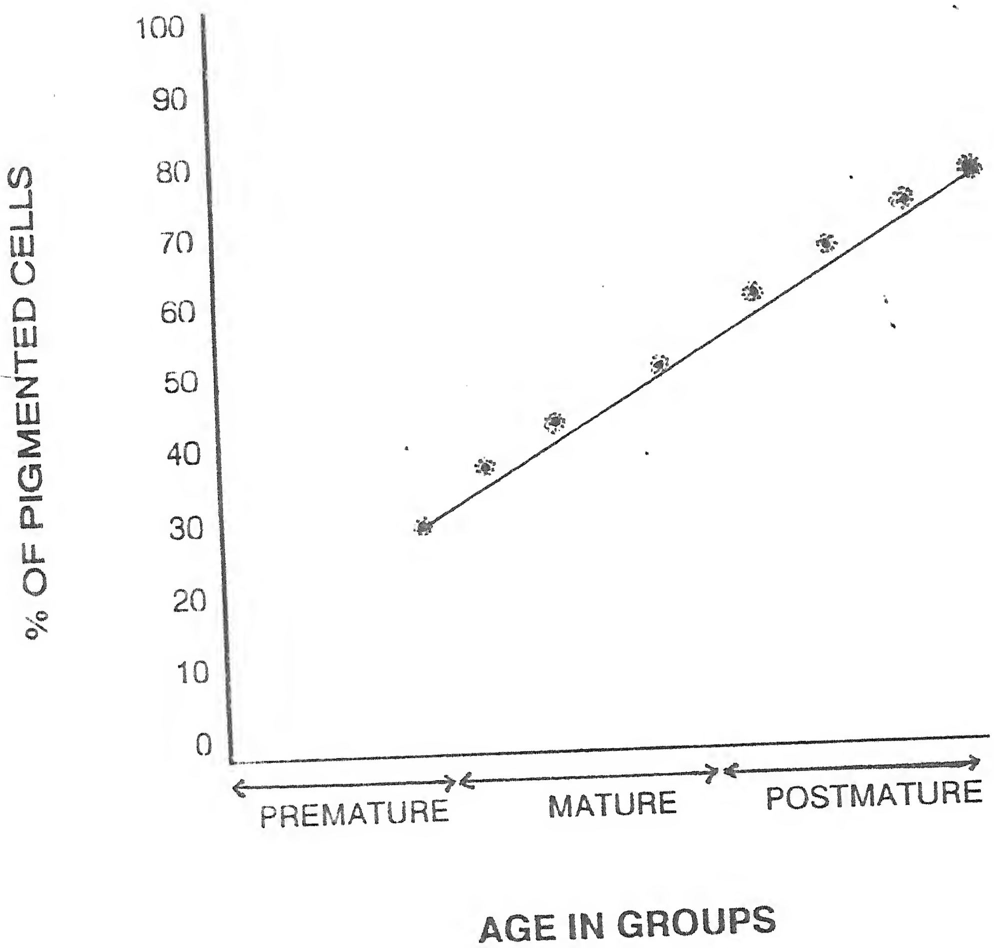

Fig. - Percentage of Pigmented Cells in Brain vs age for <u>Channa punctatus</u>

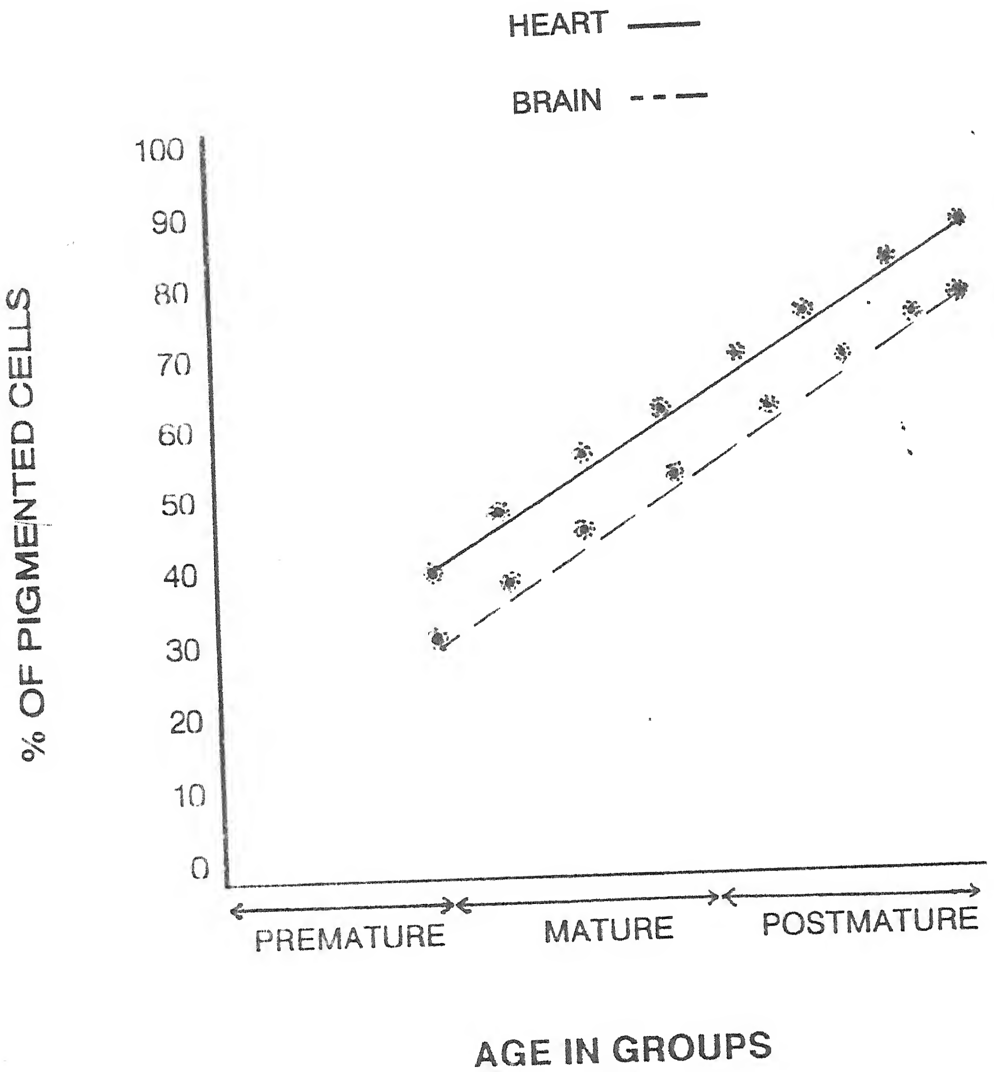

Fig. - Percentage of Pigmented Cells of Heart & Brain vs age for Channa punctatus

CHAPTER - 4

DISCUSSION

Results and conclusion of the present study on changes occurring because of aging, are based on the results and observations performed on heart and brain of Channa punctatus. How ever special attention was focused on the first appearance, distribution pattern, morphological characteristics and accumulation of lipofuscin pigments with age. Qualitative and quantitative investigations were also made in different tissues at different age levels.

For intraspecies comparisons fishes were divided in to three progressive age groups i.e. premature, mature and post mature age groups, as four age groups were made by Brody (1960) for humans, two age groups by Sharma (1967) for Ophiocephalus striatus, six age groups by Few & Getty (1967) for dogs and hogs, four age groups by Samorajski, Ordy & Reimer (1968) for mice, five age groups by Nanda & Getty (1971) for Pigs, six age groups by Vyas and Nanda (1981) for goat, four age groups by Goyal (1982) for mouse and five age groups were made by Gupta (1989) for Dysdercus similis.

For the histological and histochemical comparisons, the representative tissues from the animals at different age levels were fixed in 10 % neutral formaline solution for 3-4 days. Similarly tissues were fixed by Strehler et al (1959) in 20 % formaline for 2 days and in 10 % neutral formaline by Vyas and Nanda

(1981). Paraffin sections were cut at 6 from heart and brain as sections were cut at 10 by Brody (1960) from cerebral cortex, at 10-15 by Nandy (1968) from different parts of CNS, at 8 by Nanda and Getty (1971) from brain, at 4 by Goyal (1981) from human myocardium and sections were cut at 6 by Gupta (1983) from heart of Dysdercus similis.

In the present study sections were stained with Nile blue A, ferric ferricyanide and carbol fuchsin method for microscopic examinations. Similarly Nile blue A, sudan black B, ferric ferricyanide and carbol fuchsin stains were used by Nandy (1971), Nile blue sulphate and alcian blue by Vyas and Nanda (1981). Nile blue A and carbol fuchsin by Gupta (1989). and the sections were stained with sndan black B, alcian blue / periodic acid schiff by Goyal (1981). The stained sections were observed under light microscope for lipofuscin pigment granules. similar observations under light and electron microscope were made by Samorajski, ordy and Reimer (1968), with fluorescence microscopy by Vyas and Nanda (1981) and light microscope was used by Gupta (1983).

Results revealed that in the youngest age group one (Pre mature), no pigment deposition within the cells of heart was observed. Examinations of brain cells also failed to reveal the presence of lipofuscin pigments in the specimens of premature fishes, where as rounded and centrally placed nuclei were observed within the cells. Jayne (1950) also reported similar observation in new born

human heart. Further Brody (1960) found that the cells of neurones of a new born baby were free of lipofuscin pigments. Lipofuscin pigment was not demonstrated in heart of Dysdercus up to the age of 5 days, (Gupta 1989). Similarly lipofuscin pigment was not detected in neurones of senile guinea pigs in first two months of age (Nandy 1968) Brizzee et al (1969,1974) also failed to reveal the presence of lipofuscin in very young rat brain and in rhesus monkey brain untill the age of 3 months. According to Strehler et al (1959) no pigment was demonstrated in the heart of human beings below 10 yrs of age. Complete absence of lipofuscin particles was demonstrated in the heart of child upto the age of 6 years (Hendley et al 1963). Munnell & Getty (1968) also found that there was no pigment in the heart of dog below 6 months of age.

Absence of lipofuscin pigments in young specimens has also been describe by a number of investigators and its occurrence in the first age group has also been confirmed by several workers. Sohal(1971) has reported that there was no sign of lipofuscin pigments in the heart of housefly upto the age of 7 days. Whereas, Sohal & Sharma (1972) studied the brain of housefly and didn't find lipofuscin pigments at the age of 3 days. Goyal (1981) studied human heart and found no pigment granules in a person below 9 yrs of age. Similarly Horn et al (1981) reported the absence of pigments in adrenal gland up to 3 months old rats.

The results of the present study regarding the presence of lipofuscin at early stages of age are fully convincing and are in agreement with earlier findings of various investigators in different organism. It has a great interest to examine the first appearance of lipofuscin pigment granules. In the present investigation first appearance of lipofuscin pigment was observed in heart and brain cells of early mature fish i.e. begining of the second age group. The findings of the present study are in agreement with those of Sharma (1967), who observed first appearance and comparatively very few pigments bodies in the neurones of young animals than those of old ones. Few and Getty (1967) observed first appearance of lipofuscin pigments in the nervous system at the age of 5 months in dog and at the age of 6 months in hog. Gupta & Gupta (1983) describe that lipofuscin pigments in the mid gut of male Dysdercus appeared clearly in second age group. Nanda & Getty (1971) observed that lipofuscin pigment was present in all brain areas studied in pig but the first appearance of lipofuscin pigment was reported at the age of 1 years and two months in nucleus olivaris. Similarly Vyas & Nanda (1981) studied the nervous system in aging goat and reported the first appearance of lipofuscin in nucleus motolius nervous-trochlearis and formatic reticularis at the age of 1 year and 12 days, in the nucleus tractus mesencephalici at the age of 8 months and 16 days in nucleus motorius nervous occulomotorii at the age of 1 year 3 months and 8 days.

Goyal (1981) reported the earliest appearance of lipofuscin pigments at 9 years of age in the left ventricular myocardium. Similar observations were also made by Whiteford & Getty (1966) in canine and porcine brain. According to them, hypoglossal nucleus in canine exhibited evidence of lipofuscin pigments at 2.5 years of age whereas in inferior olivary nucleus, the accumulation of pigments appeared at the age of 4 years. Similarly in porcine brain hypoglossal nucleus showed the first evidence of lipofuscin accumulation at the age of 3 years and 4 months and in inferior olivary nucleus at the age of 4 years.

Munnell and Getty (1968) have reported the first appearance of pigments in the human myocardium after 10 years. Munnell and Getty (1968) also compared the period of dog life with that of human life, they were of the opinion that 6 months to 1 year, period of dogs life can be compared to the 10-20 years period of human. The periods are comparable only when the sexual maturity taken in to consideration in both dog as well as in human. In the present study age groups were also made on the basis of sexual maturity of fishes.

In the present investigation it was found that the first appearance of lipofuscin pigment is same in both the organs of the fishes i.e. the heart and brain.

In the second age group it was observed that the process of pigments accumulation in heart and brain gradually increased. Staining with Nile blue A, carbol fuchsin and ferric ferricyanide methods, pigments were first observed as scattered granules in some cells of heart and brain in the begining of second age group. These findings may be corelated with the results of Brizzee et al (1974) who observed scattered granules in only some of the neurons of the inferior olive of primate at 6 months of age.

Cells of the heart and brain of early mature fishes were found to contain few lipofuscin pigment granules distributed in the cytoplasm near the nuclei. This is in agreement with the findings of Few & Getty (1967) in dog and hog. Goyal (1982) also reported that nerve cells of mouse at 5 months of age contained very few pigments. The pigments appears to increase linearly with age. Such linearity between pigment granules and age was also observed by Munnell and Getty (1968) in heart of aging canine. Gupta (1984) observed linear accumulation of lipofuscin pigments in midgut of Dysdercus similis, and Patro et al (1992) also observed linear increase of lipofuscin accumulation in myocardial cells of albino rats.

With different stains lipofuscin was frequently observed in heart as well as in brain cells. Lipofuscin pigments stained by Nile blue A method appeared as

dark blue green granules. Similar observations were made by Sharma (1967) who reported that lipofuscin pigments appeared as dark brown granules in fishes, hemiductylus and natrix. Similarly Reichel et al (1968) observed that lipofuscin pigments appeared as yellow orange granules in rodents brain. Whiteford and Getty (1966) observed lipofuscin pigments as blue green granules in the neurones of canine and porcine brain. Nandy (1971) also observed lipofuscin pigments as green yellow granules in younger mice.

The morphological characteristics of lipofuscin pigments in the heart and brain were found to be very similar in the mature age group. Similar observations were made by Hess (1955) in neurones of man and small laboratory animals. Miquel (1971) was of the opinion that lipofuscin pigment granules in insects were similar in colour and size to the mammalian lipofuscin pigments and other common characteristics of mammalion and insects aging seems to be the accumulation of age pigments. The fine structure of lipofuscin granules in early age was described by Malkoff and Strehler (1963) in human heart. Christensen (1965) and Frank & Christensen (1968) in guinea pig interstitial cells of leydig and Fawcett & Bargos (1960) in the interstitial cells of human testes.

In the fishes of mature age group pigment granules were observed rounded in shape and homogeneous in structure. The lipofuscin pigments appeared as a single granule scattered irregularly throughout the cytoplasm.

These morphological findings are in agreement with the findings of Totaro and Pisanti (1980 a) who studied small rounded and homogeneous and scattered lipofuscin pigment granules in electric lobes of young torpedo.

In the heart cells of fishes ranging in mature age group, the pigmentation usually started as tiny granules diffusely distributed in the cytoplasm. Similar observations were made by Nandy (1968, 1971) who reported diffusely distributed granules in the neurones of guinea pig at the age of 6 months and in neurones of young mice ranging in age from 3 to 5 months. According to Totaro and Pisanti (1979) the pigment granules appeared as tiny and diffusely distributed in newborn torpedo.

In the present study it was observed that as age increased, a moderate increase in the number of pigmented cells and pigment granules percells and maximum concentration of lipofuscin pigment was observed in the heart cells. Similar observations were made by Donato et al (1979) in housefly. Goyal (1981) also reported that the number of pigmented cell and pigment granules had increased in human myocardium and continued to increase in number and started accumulating gradually with age. According to Brody (1960) as one proceeds through adult age group there is sharp decrease in the number of cells free from pigments and an increase in the number of cells containing pigments in human cerebral cortex. While Chu, (1954) found that in the human

spinal cord the amount of pigments increases with age of the individuals and Wilcox (1959) after examination of the cranial nerves in guinea pigs, also stated that the accumulation of lipofuscin is corelated with aging.

In present investigations a greater number of dark granules become prominent and appeared to be larger in size in the heart and brain. This may be corelated with the findings of Few & Getty (1967), who reported that before function, the initial pigment bodies appeared to enlarge as their substructure become denser in nurons of hogs and dogs. Totaro and pisanti (1980 b) reported that in younger animals, the granules are quite small, on the other hand, pigment was clearly seen in neurones of adult torpedo, these characteristics seems to evolve with age.

In heart and brain cells of mature fishes the pigment granules lie scattered in the cytoplasm, but occasionally they show a tendency to aggregate, therefore few pigment granules were observed quite close to each other in late mature fishes. Similar findings were made by Sharma (1967) in fishes and Goyal (1981) in man. Nanda and Getty (1971) demonstrated that nucleus olivaris inferior nucleus olivaris of aging pig showed a tendency for lipofuscin accumulation at the age of 3 years and 9 months.

In the present study although exact quantitative evaluation was not practical, results of attempts to corelate the amount of pigments occurring in the

neurones and cardiac muscles with age indicate that in general pigmentation increases with age with respect to both the number of cells affected and concentration per cell. It is of perticular interest to estimate the percentage of intracellular volume which is occupied by pigments. The percentage of pigmented cells in different tissues progressively increased with age. In present investigation the pigmented cells was observed approximately 40-60 % from heart and 30-50 % from brain cells of mature fishes. Similar observations were made by Nanda & Getty (1971) in aging pig, they reported that at the age of 3 years and 9 months, nucleus olivaris inferior cells were pigmented 40-50 % from nucleus hypoglossus 60-70 % and dorsal motor nucleus of vagus about 35-40 % at the same age. Samorajski et al (1968) reported that 55 % of dorsal ganglion cells and 47 % of purkinge cells in aging mice were pigmented at 8 months of age. According to whiteford and Getty (1966) cochlear nuclei contained 40-50 % of pigmented cells at 7-8 years of age where as 12-34 % of mesencephalic nucleus of the trigerminal nerve cells were pigmented at the age of 7-8 years respectively.

In the present study it was also observed that the rate of pigments accumulation is also affected by environmental factors. Data indicates that the rate of pigments accumulation is comparatively low in cultured habitat than that of natural habitat. Similar observations were also made by Papafrangos and Lyman (1982) in mesocricetus (Hamster). They indicate that the rate of lipofuscin accumulation in the hibernators is slower than that in the nonhibernators.

The third age group includes postmature fishes. The observations of heart and brain cells of post mature fishes revealed variations in size and shape. This may be correlated with the findings of Hasan and Gless (1973) who reported that pigment granules revealed variations in shape and size and electron density with increasing in age. In the cells of heart and brain of post mature fishes it was observed that two or more pigment bodies fused in several instances to form larger pigment granules. Similar observations were made by Patro et al (1992), who reported that the size of the pigment bodies tended to increase with increased age and deposition of pigments, according to them, in the myocardial cells of young animals the pigment bodies were mostly in the form of granules measuring 1.25 m in diameter and those found in the cells of the adult and senile animals had a greater tendency towards the formation of loose aggregates and duplexes and the average size of the pigments particulates increased to 3.95-5.43 m in the adult and senile myocardial cells respectively.

In the present investigation as the fish advanced in age the pigment granules appeared dense and heterogeneous in structure. Similar observations were made by Few and Getty (1967) in hogs and dogs. Brizzee (1969) also reported that the predominantly scattered fluorescent bodies were found in young and adult, and the tendency of these material to accumulate in clusters was reported in perikaryon in aged rat.

In addition, the dense bodies were more heterogeneous and become more irregular in the older animals, there was a tendency for these pigments to collect in to groups throughout the cytoplasm. The intracellular pigments distribution is in the agreement with the findings of Muhlmann (1910) who noted that the homogeneous distribution of pigment granules in ganglion cells of the guinea pigs and man was gradually lost with increasing age, gathering in clusters mass which continued to increase in size. Similar observations were also made by Samorajski et al, (1964) in neurones of adult human beings.

In the post mature fishes the majority of the heart and brain cells contained pigment granules. However greater number of these pigmented cells contain scattered pigments and only a relatively small number showed clumped pigments in the cytoplasm. This is in agreement with the findings of Brody (1960) who reported that cortical cells in human brain contain scattered pigments as well as clumped pigments in the cytoplasm. The number of cells containing pigments increases with increasing age. Goyal (1981) is of the opinion that pigment bodies appeared in accumulated and concentrated masses in human myocardium in the fifth decade. Munnell & Getty (1968) also revealed the clumping of lipofuscin granules in dog with the advancement of age.

It was clearly observed in the heart and brain cells that the size, complexity and intracellular distribution of these pigments was variable to some

extent among different cells even of the same section. In the heart most of the granules occupied the entire cytoplasmic area, while few of them were deposited at the periphery of cells. In brain cells, the pigment granules generally distributed in clusters around the nucleus. Similar observations were made by Jayne (1950) and Hasan & Glees (1973), who revealed variations in size, shape and distribution of pigment bodies among different cells even of the same section. Samorajski et al, (1968) reported that dark pigment bodies appeared to be larger in size and were often concentrated within the peripheral portion of perikaryon in aging mice.

In present investigation all the four categories of cells were found in the cells of heart and brain of post mature fishes, i.e. cells without pigment, cells with few pigments, cells containing homogeneous pigments and cells containing heterogeneous or clusters of pigments granules distributed throughout the cytoplasm. Similar observations were made by Nandy (1971) who reported the following three categories of cells in aging mice i.e. cells with no pigment, cells with few diffuse pigments and cells showing heavy (clumpy) pigmentation. Similarly Gupta (1985) also reported all categories of cells in the heart cells of Dysdercus similis during the investigation of fourth age group.

In the present study the pigment granules and pigmented cells increased linearly with age. Similar observations were made by Strehler et al, (1959) who reported that lipofuscin granules increased linearly in cardiac

muscles fibres throughout life. Similar corelation of increased accumulation of fluorescence with age have been shown by Sheldahl & Tappal (1974) in aging Drosophila.

In present investigation, heavy deposition of pigments ware observed in heart where as brain cells show comparatively low deposition of lipofuscin granules. These results are in agreement with the findings of Brody (1960), who reported the highest percentage of pigmented cells in the precentral gyrus and the lowest percentage in striate cortex of old human brain. While Donato and Sohal (1978) noted that the greater number of fluorescent granules appeared within the epithelial cells of the midgut where as heart and malpighian tubule contained less fluorescent granules in male houseflies ranging in age from 15 to 25 days but, Papafrangos et al (1982) have also reported more pigment bodies in the brain tissue than in the heart tissues of hamsters.

In present study maximum percentage of pigmented cells was observed in different tissues of post mature fishes. The percentage of pigmented cells in heart was 60-85% and in brain 50-75% cells were pigmented. The highest percentage of pigmented cells was noted in heart. These results are in agreement with the findings of Nanda and Getty (1971) in the nervous system of aging pig. According to them 80-90 % of purkinje cells of cerebellar cortex and about 75-85 % of neurones were pigmented by 10 years of age. Samorajski et al (1968)

described that 85 % of dorsal ganglion cells and 98 % of purkinje cells of aging mice were pigmented by 30 months of age.

The present observations indicate that lipofuscin pigment was present in all the tissues studied. The amount of pigments as well as percentage of pigmented cells increased with age. The microphotographs show that the pigmentation in heart and brain increases progressively with age. The linear relationship of accumulation of lipofuscin with age has been established and statistically significant positive corelations was found. Strehler et al (1969) in man and Munnell and Getty (1968) in canine also established similar relationship.

In the present investigation Nile blue A, carbol fuchsin and ferric ferricyanide methods were used for histochemical studies of lipofuscin pigments in heart and brain of Channa punctatus in different age groups.

It is very interesting to note that lipofuscin pigments in the cells of the heart and brain in different age groups reacted strongly to lillie's alternative Nile blue A method. This in in agreement with the findings of Sharma (1967) who reported that pigment granules in the nerve cells of Rana, Bufo, Uromastix and Natrix reacted strongly with Nile blue A. Totaro et al (1981) were of the opinion that lipofuscin pigments in CNS of merine teleostei Sargus. s. and Scorpoena. s. had strong affinities with lillie's alternative Nile blue A method.

The lipofuscin pigments in the heart and brain exhibit variations in the staining properties in mature and post mature fishes, as pigments in different tissues of mature fish reacted moderately to Schomorl's ferric ferricyanide method, whereas old tissues had strong affinities to this stain. These findinds are similar to those of Nandy (1971) in neurones of young old mice. He reported that in younger animals, lipofuscin pigments were more easily stained with sudan black B and PAS while on other hand, in older animals, it was easily stained with Nile blue A and ferric ferricyanide method.

Totaro et al (1981) also described that pigments had characteristically different staining behaviour in marine teteostei. According to them in central nervous system of Surgus s. and Scorpoena s. lipofuscin exhibited a moderate positivity in Schmorl's method whereas in Coris j. lipofuscin reacted mildly. Totaro at al (1981) also reported major variations in staining properties in myocardium of merine teleostei.

The lipofuscin in the heart and brain showed great variations in the staining properties with carbol fuchsin. In the second age group the pigment granules in heart related moderately whereas in the brain exhibits a mild positivity while in, third age group both the tissues reacted moderately. This is in agreement with the findings of Nandy (1971) in neurones of young and old mice. According to Sharma and Manocha (1977) the pigments showed

characteristically different staining behaviour as one granule may be moderately or intensely positive to a particular stain and the other lying in its vicinity may be negative or partly negative and partly positive. Manocha & Sharma (1978) were also made similar observations in spinal cord of squirrel monkey.

It is of great interest that in all the tissues of young fishes, lipofuscin pigments were more easily stained with Nile blue A, than that of ferric ferricyanide and carbal fuchsin methods. On the other hand pigment granules in the heart and brain in older fishes reacted moderately with all the stains used. These variations may be due to differences in the composition of lipofuscin pigments. The findings of Sharma (1967) in cold blooded vertebrates, Nandy (1971) in mice and Totaro et al (1981) in merine teleostei showed similar results. Gupta & Gupta (1985) reported that in midgut of male Dysdercus in age group second, the pigment granules appeared in the cytoplasm cleared with Nile blue A than ferric ferricyanide they were of the opinion that pigment granules in older age reacted with both the stains. Strehler (1964) reported that a typical section stained with Sudan black B is essentially similar in appearance to the section stained with Nile blue sulphate.

It is evident from the present study that the affinities of lipofuscin with different stains varied cansiderably in different age groups, and these variations may be due to different composition of lipofuscin pigments at different age

levels.

As such, it is very clear from the findings of the present study that the accumulation of lipofuscin pigments gradually increased with age. The statistical analysis of data further confirms the fact that the accumulation of lipofuscin is an age oriented.

CHAPTER - 5

SUMMARY

Objectives of the present investigations were to determine the first appearance of lipofuscin pigments, its occurrence, the presence of pigmented cells per volume, distribution, morphological characteristics and staining properties of lipofuscin pigment in the heart and brain of Channa punctatus at various age groups. Qualitative and quantitative investigations were also made in different tissue in different age groups. It was also observed that the time of the appearance of pigments and its subsquent increase varied in different organs at different age levels. Both the tissues i. e. heart and brain were examined in three progressive age groups. i. e. premature, mature and post mature respectively.

Results of the present study revealed that the cells were found without lipofuscin pigments in heart and brain of premature fishes. The lipofuscin pigments were first observed in the cells of heart and brain of early mature fish or at the beginning of second age group. An interesting finding of present investigation is that the pigment granules and pigmented cells increased progressively with age of fish. In the begning of the second age group the pigment granules were observed to be very few in number, homogeneous in structure and irregularly distributed within the cells of the heart and brain. The pigments were also observed so abundant that they often occupied the entire

cytoplasmic area of heart cells where as, in brain less accumulation of pigment granules were noted.. A tendency of pigment granules to aggregate in to the groups were also observed in the heart and brain in second age group.

The complete aggregation of pigment granules were observed in the third age group. As the fishes increased in age the granules enlarged and tended to form clusters. The pigment granules appeared to be larger in size and the intracytoplasmic location of pigment granules generally varied with age. The granules usually occupied the entire cytoplasmic area of the cells and few noted peripheral in position. Heterogeneous lipofuscin pigments were clearly observed in this age group. As compared to the heart accumulation of lipofuscin pigment granules in brain was noted to be slow. All the four categories of cells were observed in the cells of heart and brain of fishes of third age group.

Results revealed that accumulation of lipofuscin pigment was found to increase upto third age group in all the tissues examined. As pigmentation progressed more and more granules appeared to form clusters. In heart and brain mojority of cells contained clumped pigments instead of scattered granules in cytoplasm. The distribution of pigment was not uniform in both the tissues i. e. most of the pigment granules were found to scattered in the cytoplasm near the nuclei, while there were only few pigments deposited at the periphery. Different sizes and variable amounts of pigment granules were recorded in heart and brain

at different age levels in all the age groups. The pigment granules in brain cells were comparatively smaller in size than the pigments of heart cells. Heavy pigmentation was also observed in the heart and least pigmentation in brain, The counting of pigmented cells in heart and brain helped to obtain the mean percenatge of cells.

Results revealed that the highest percentage of pigmented cells was found in heart and lowest in brain. Secondly the pigmented cells increased progressively in all the tissues. Although lipofuscin occurs in all the tissues studied but the highest concentration of lipofuscin granules was found in heart than in the brain cells.

Table - 1

Summary of the pigmented cells in different tissues with age.

Tissues	Age groups	Pigmented cells/ volume	Cells pigmented
		Mean ± S.E.	%
Heart	Premature	-	-
	Mature		
	a)	8 ± 0.37	40
	b)	10 ± 0.61	50
	c)	11 ± 0.47	55
	d)	12 ± 0.23	60
	Post mature		
	a)	13 ± 0.42	65
	b)	14 ± 0.36	70
	c)	16 ± 0.52	80
	d)	17 ± 0.26	85
Brain	Premature	-	-
	Mature		
	a)	6 ± 0.18	30
	b)	8 ± 0.29	40
	c)	9 ± 0.86	45
	d)	10 ± 0.63	50
	Post mature		
	a)	12 ± 0.26	60
	b)	13 ± 0.38	65
	c)	14 ± 0.54	70
	d)	15 ± 0.73	75

Histochemical observation of the heart and brain of aging Channa punctatus revealed that the pigment granules showed characteristically different staining behaviour. These variations may be due to differences in the composition

of lipofuscin pigments. In the present investigation it was clearly noted that lipofuscin pigment granules in the heart and brain of mature and post mature fishes were easily stained Lillie's Nile blue A method, thus showing a strong positivity to the pigment granules.

Schomorl's ferric ferricyanide stain reacted moderately to the pigment granules of the heart and brain in mature fishes. Pigment granules in different tissues of post mature fishes, however, reacted strongly to ferric ferricyanide stain.

Histochemical nature of lipofuscin pigments for carbol fuchsin was observed to some what different. Lipofuscin pigments in heart and brain of mature fishes exhibited mild and moderate positivity to Long - Ziehl - Neelsen's carbol fuchsin method while in post mature fishes granules were moderately reacted to carbol fuchsin in both the tissues.

The findings of the present study reveal significant relationship of lipofuscin with the age of Channa punctatus.

Lipofuscin pigment is supposed to be a parameter of age as age pigment or lipofuscin pigment progressively increased with age of the fishes.

In premature fishes cells were found with no pigment granules .

The first appearance of lipofuscin pigments was observed at the begining of mature age.

Pigment granules per cells and pigmented cells, increase with increasing age of fish and vary at different age levels.

Homogeneous pigment granules were observed in mature age group.

Heterogeneous pigment granules were observed in post mature fishes. The maximum concentration of pigment granules was observed in the heart cells of post mature fishes. There after mortility began in fishes.

It was observed that accumulation of lipofuscin pigment became saturated in old age and this stage supposed to be the fatal age of fishes.

Differences between young and old pigment granules were clearly observed in mature and post mature fishes, and histochemical observations showed that lipofuscin pigment granules had different affinities with different stains at various age groups.

CHAPTER - 6

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